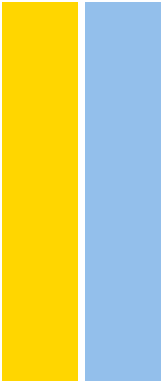


Deciphering horizontal transmission of resistance in lung cancer: The role of caveolae-dependent endocytosis

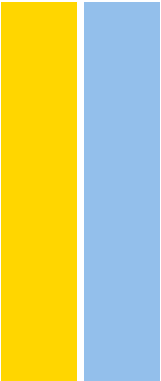
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2024



Deciphering horizontal transmission of resistance in lung cancer: The role of caveolae-dependent endocytosis

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LUANA BEATRIZ REIS PAIS PEREIRA

**DECIPHERING HORIZONTAL TRANSMISSION OF RESISTANCE IN
LUNG CANCER: THE ROLE OF CAVEOLAE-DEPENDENT
ENDOCYTOSIS**

Dissertação de Candidatura ao grau de Mestre em Oncologia
– Especialização em Oncologia Laboratorial – submetida ao
Instituto de Ciências Biomédicas de Abel Salazar da
Universidade do Porto.

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e Inovação em Saúde (i3S)

Agradecimentos

Em primeiro lugar, quero agradecer ao meu orientador, Professor Doutor José Carlos Machado, por me proporcionar a oportunidade de desenvolver a minha dissertação de mestrado neste projeto que me cativou desde o primeiro momento. Obrigada pelos ensinamentos e por estar sempre disponível para me ajudar.

Quero também agradecer ao meu “co-orientador”, José Guimarães, por ter ouvido e respondido a todas as minhas perguntas sempre com paciência (e ambos sabemos que foi preciso muita). Obrigada por tudo! (Embora sinta que poderia ter interiorizado mais se falasses mais alto...)

O meu muito obrigada à grande amiga que fiz neste mestrado, Madalena Corado. Obrigada pelas conversas, distrações e por puxarmos sempre uma pela outra. Tornaste este percurso mais leve e suportável.

Ao meu namorado, João Pacheco, obrigada por estares sempre do meu lado e me fazeres feliz todos os dias. Das tuas muitas qualidades, a mais valiosa para esta dissertação foi, sem dúvida, seres Mestre em Bioinformática e me teres ajudado com o RStudio.

Por fim, quero agradecer à minha família. À minha Mãe, ao meu Pai e à minha irmã, obrigada pelas palavras de encorajamento e por tudo o que fizeram e continuam a fazer por mim e pela minha felicidade. E um grande obrigada ao meu avô Reis, de quem tantas vezes me lembrei, recordando-me do significado desta etapa e dando-me forças para continuar.

Abstract

Newly diagnosed stage IV lung adenocarcinoma (LADC) patients are subjected to molecular testing in order to identify activating mutations in their tumors that allow the treatment with targeted therapies. When these mutations occur in the ATP binding pocket of the tyrosine kinase domain of the epidermal growth factor receptor (EGFR), the tumor is sensitive to tyrosine kinase inhibitors (TKIs), namely erlotinib. However, new mutations can arise, such as the p.T790M mutation in exon 20 of *EGFR*, and result in EGFR-TKI therapy resistance. Although third-generation inhibitors are now available (e.g., osimertinib) to treat these resistant tumors, the mechanisms by which resistance emerges are not yet completely understood.

Previous results from our group showed that, in patients with relapsing tumors, the allelic frequency of erlotinib sensitizing mutations is higher than that of the p.T790M resistance mutation. Hence, we defined that these tumors have a resistance phenotype, since they contain few tumor cells with resistance mutations while the majority of the tumor cells display a resistance phenotype with no common resistance mutations in their genotype. Additionally, we found that these tumors exhibit an upregulation of genes associated with the endocytosis pathway, mainly *CAV1*. This led us to hypothesize that the tumor cells that exhibit the resistance phenotype acquire it through a mutation-independent mechanism, via the secretome or via extracellular vesicles, from tumor cells with resistance mutations. We designated this process “horizontal transmission of resistance”. As such, the main aim of this project was to explore how the overexpression of *CAV1* contributes to the development of resistance to EGFR-TKI therapies in LADC.

The kinases PI3K, AKT and ERK were found upregulated in tumors displaying this resistance phenotype. Therefore, the dual inhibition of these kinases and EGFR (by administering erlotinib) was evaluated using MTT assays. The PI3K and ERK inhibitors, when combined with erlotinib treatment, rescued sensitivity to the EGFR-TKI in LADC cells that exhibit the mentioned resistance phenotype, confirming that indeed these molecules are highly expressed in resistant tumors and that the EGFR-PI3K or EGFR-ERK dual inhibition might represent a way to extend the effectiveness of the targeted therapy.

Moreover, since this erlotinib-resistant phenotype was a stable characteristic not caused by commonly observed resistance mutations, we decided to analyze and compare epigenetic signatures between LADC cells before and after acquiring the resistance phenotype. Hence, through a DNA methylation array, we found that there are differences in the DNA methylation patterns of LADC cells before and after acquiring the resistance phenotype.

In conclusion, resistance to targeted therapies in lung cancer is not solely driven by genetic mutations. Intercellular communication coupled with epigenetic alterations, such as changes in DNA methylation patterns, may play a significant role in the development of therapy-resistant phenotypes. Although our results are promising and provide new insights into the underlying mechanisms of resistance, further experiments are needed to validate these conclusions and explore the way for alternative approaches to overcome therapy resistance in LADC.

Keywords: Lung adenocarcinoma, EGFR, erlotinib, therapy resistance, DNA methylation

Resumo

Pacientes recentemente diagnosticados com adenocarcinoma do pulmão em estadio IV realizam testes moleculares de modo a identificar mutações ativadoras nos seus tumores que permitam o tratamento com terapias-alvo. Quando estas mutações ocorrem no local de ligação ao ATP do domínio tirosina cinase do recetor do fator de crescimento epidérmico (EGFR), o tumor é sensível a inibidores da tirosina cinase (TKI), nomeadamente ao erlotinib. No entanto, podem surgir novas mutações, como a mutação p.T790M no exão 20 do EGFR, que resultam em resistência aos EGFR-TKIs. Embora atualmente estejam disponíveis inibidores de terceira geração (ex.: osimertinib) para tratar estes tumores resistentes, os mecanismos pelos quais a resistência surge ainda não estão totalmente compreendidos.

Resultados anteriores do nosso grupo de investigação mostraram que, em doentes com tumores recidivantes, a frequência alélica de mutações sensíveis ao erlotinib é superior ao da mutação de resistência p.T790M. Por conseguinte, definimos que estes tumores têm um fenótipo de resistência, uma vez que contêm poucas células tumorais com mutações de resistência, enquanto a maioria apresenta um fenótipo de resistência (o seu genótipo não apresenta mutações de resistência frequentemente observadas). Além disso, verificámos que estes tumores apresentam uma sobre-expressão de genes associados à endocitose, principalmente da CAV1. Isto levou-nos a hipotetizar que as células tumorais que exibem o fenótipo de resistência o adquirem através de um mecanismo independente de mutações, através do secretoma ou de vesículas extracelulares, provenientes de células tumorais com mutações de resistência. Este processo foi designado por nós de “transmissão horizontal de resistência”. Assim, o principal objetivo deste projeto foi explorar como é que a sobre-expressão de caveolina contribui para o desenvolvimento de resistência a EGFR-TKIs em adenocarcinoma do pulmão.

As cinases PI3K, AKT e ERK encontram-se sobre-expressas nos tumores que apresentam este fenótipo de resistência e, por isso, a inibição dupla destas cinases e do EGFR (através da administração de erlotinib) foi avaliada utilizando ensaios MTT. Os inibidores da PI3K e ERK, quando combinados com erlotinib, recuperaram a sensibilidade ao EGFR-TKI das células de adenocarcinoma do pulmão que exibem o fenótipo de resistência mencionado, confirmando que, de facto, estas moléculas estão sobre-expressas em tumores resistentes e que a dupla inibição EGFR-PI3K ou EGFR-ERK pode representar uma forma de prolongar a eficácia do tratamento dirigido.

Além disso, uma vez que este fenótipo de resistência ao erlotinib é uma característica estável não causada por mutações de resistência frequentemente observadas, decidimos analisar e comparar o perfil epigenético das células tumorais antes e depois de adquirirem

o fenótipo de resistência. Assim, através de um *array* de metilação do ADN, verificámos que existem diferenças nos padrões de metilação do ADN das células tumorais antes e depois de adquirirem o fenótipo de resistência.

Em suma, a resistência às terapias-alvo no cancro do pulmão não é apenas causada por mutações genéticas. A comunicação intercelular associada a alterações epigenéticas, como alterações nos padrões de metilação do ADN, pode desempenhar um papel no desenvolvimento do fenótipo de resistência à terapia. Embora os nossos resultados sejam promissores e forneçam mais conhecimento sobre mecanismos de resistência, são necessárias mais experiências para validar estas conclusões e explorar estratégias alternativas para contornar a resistência ao tratamento no adenocarcinoma do pulmão.

Palavras-chave: Adenocarcinoma do pulmão, EGFR, erlotinib, resistência à terapia, metilação do ADN

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List of Abbreviations

LADC	Lung Adenocarcinoma
EGFR	Epidermal Growth Factor Receptor
TKI	Tyrosine Kinase Inhibitor
DNA	Deoxyribonucleic Acid
PI3K	Phosphatidylinositol-3-Kinase
AKT	Protein Kinase B
ERK	Extracellular signal-Regulated Kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
SCLC	Small Cell Lung Cancer
CT	Computed Tomography
PD-L1	Programmed Death-Ligand 1
ctDNA	Circulating tumor DNA
NSCLC	Non-Small Cell Lung Cancer
GGN	Ground Glass Nodule
AAH	Atypical Adenomatous Hyperplasia
AIS	Adenocarcinoma <i>In Situ</i>
MIA	Minimally Invasive Adenocarcinoma
ILA	Invasive Lung Adenocarcinoma
AT2	Alveolar Type II cells
KRAS	Kirsten Rat Sarcoma Virus
ALK	Anaplastic Lymphoma Kinase
ROS1	ROS proto-oncogene 1
RET	Rearranged during Transfection proto-oncogene
CNV	Copy Number Variation
RTK	Tyrosine Kinase Receptor
ERBB	Erythroblastic leukemia viral oncogene
ATP	Adenosine Triphosphate
RAF	Rapidly Accelerated Fibrosarcoma
MAPKK/MEK	Mitogen-Activated Protein Kinase Kinase
mTOR	Mammalian Target Of Rapamycin
STAT	Signal Transducers and Activators of Transcription
BRAF	V-Raf murine sarcoma viral oncogene homologue B1
MET	MET proto-oncogene receptor tyrosine kinase

CD74	Cluster of Differentiation 74
SDC4	Syndecan 4
NTRK	Neurotrophic Tyrosine Receptor Kinase
NRG1	Neuregulin-1
SABR	Stereotactic Ablative Body Radiotherapy
CRT	Chemoradiotherapy
PD-1	Programmed Death-1
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
CD80/86	Cluster of Differentiation 80/86
FDA	Food and Drug Administration
PFS	Progression-Free Survival
OS	Overall Survival
EMA	European Medicines Agency
CDK	Cyclin-Dependent Kinases
RB	Retinoblastoma
E2F	E2F transcription factor
PCR	Polymerase Chain Reaction
NGS	Next-Generation Sequencing
NRAS	Neuroblastoma Rat Sarcoma viral oncogene homolog
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic subunit Alpha
AF	Allelic Frequency
CM	Conditioned Media
RNA	Ribonucleic Acid
ESS	Erlotinib-Sensitive S tumors
ERS	Erlotinib-Resistant S tumors
CAV	Caveolin
FC	Fold Change
FDR	False Discovery Rate
HGT	Horizontal Gene Transfer
EV	Extracellular Vesicle
GSK-3	Glycogen Synthase Kinase 3
RSK	Ribosomal S6 Kinase
FBS	Fetal Bovine Serum
FFPE	Formalin-Fixed, Paraffin-Embedded

MOI	Multiplicity Of Infection
shRNA	Short hairpin Ribonucleic Acid
tGFP	Turbo Green Fluorescent Protein
EDTA	Ethylenediamine Tetra-acetic Acid
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
IC50	Inhibitory Concentration 50%
DMSO	Dimethyl Sulfoxide
SD	Standard Deviation
ANOVA	Analysis Of Variance
FITC	Fluorescein Isothiocyanate
PCA	Principal Component Analysis
PC1/2	Principal Component 1/2
DMP	Differentially Methylated Position
R²	Coefficient of determination
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRi	Clustered Regularly Interspaced Short Palindromic Repeats interference

Introduction

A neoplasm, or cancer, is a group of diseases that arise due to the uncontrollable division of cells triggered by gain-of-function mutations in proto-oncogenes and/or loss-of-function mutations in tumor suppressor genes. The cells' deoxyribonucleic acid (DNA) repair machinery does not recognize these errors and so they are transmitted to the daughter cells and perpetuated, leading to the growth of a malignant tumor ("Neoplasia," 2018).

When mutations that confer selective advantages (e.g., proliferation, survival) occur in lung cells, lung cancer can quickly develop ("Neoplasia," 2018).

1. Lung Cancer

1.1. Epidemiology

According to the most recent Global Cancer Observatory data (GLOBOCAN, 2022), that dates to 2022, lung cancer is the most diagnosed cancer worldwide, corresponding to 12.4% of total cancer cases, and it has the highest mortality rate in the world, being responsible for 18.7% of total cancer deaths.

Despite 63.1% of lung cancer cases and 62.9% of lung cancer deaths occurring in Asian patients, Europe is the second most affected continent by this disease. In Portugal, lung cancer ranks fourth in incidence (8.8% of cancer cases), yet it is the deadliest cancer (15.0% of cancer deaths).

1.2. Risk Factors

The major risk factor contributing to these numbers is tobacco smoking (Bade & Dela Cruz, 2020), with the smoking intensity, i.e., the number of cigarettes smoked per day and the lifetime duration of smoking, contributing proportionally to the risk (Nasim et al., 2019; Schabath & Cote, 2019).

However, lung cancer is increasingly being diagnosed in people who never smoked, defined as individuals who smoked less than 100 cigarettes throughout their lifetime (Pomerleau et al., 2004). In fact, and acknowledging that there are geographic differences, 10-15% of lung cancer cases are estimated to occur in never-smokers (Shehata et al., 2023). Risk factors that can contribute to non-smoking related lung cancer include second-hand exposure to tobacco smoke, which is responsible for 17% of lung cancer cases in never-smokers, indoor (e.g., wood and coal burning) and outdoor air pollution, occupational

exposures (e.g., radon gas, asbestos), chronic lung diseases (e.g., chronic obstructive pulmonary disease (COPD)), infections (e.g., by the human papillomavirus) and genetic predispositions (Bade & Dela Cruz, 2020). Moreover, this non-smoking related lung cancer usually are adenocarcinomas that occur in young people and women (Devarakonda et al., 2015), whilst smokers are typically diagnosed with small cell lung cancer (SCLC) and squamous cell carcinoma (Ruiz-Cordero & Devine, 2020).

1.3. Clinical Manifestations

In its early stages, lung cancer is an asymptomatic disease, which makes it difficult to diagnose and further treat with a curative intent. Symptoms typically emerge late, as the disease progresses, and tend to be non-specific, being the most common ones: persistent cough, fatigue, weight loss, dyspnea, hemoptysis, chest pain and hoarse voice. These clinical manifestations can already be related with tumor invasion of surrounding tissues (Alexander et al., 2020; Nasim et al., 2019).

1.4. Diagnosis

The diagnosis of lung cancer involves imaging tests complemented with the tissue's pathological evaluation (Alexander et al., 2020; Nooreldeen & Bach, 2021).

1.4.1. Low Dose Computed Tomography Scans

Low dose computed tomography (CT) scans are used for lung cancer screening in high-risk individuals, i.e., 55-74 years old patients with 30 pack-years or more (pack-years = number of cigarette packs smoked per day × the number of years smoked) that currently smoke or that quit in the last 15 years. Spiral/Helical CT scans can detect lesions in the peripheral lung as small as 1-5 mm of diameter, nevertheless the sensitivity of this imaging test for central tumors is significantly lower (Nooreldeen & Bach, 2021). Because of this and other issues, namely a 23.3% false positive rate leading to over-diagnosis, exposure to radiation (though less than with conventional CT) and high cost, additional tests are required to confirm a positive result (Ning et al., 2021).

1.4.2. Biopsy

Endobronchial ultrasound (EBUS) combined with transbronchial needle aspiration (TBNA) enables a real-time ultrasound-guided harvesting of malignant tissue from centrally located primary tumors and of lymph nodes. For tumors located in the peripheral lung,

image-guided transthoracic needle biopsies are usually performed (Alexander et al., 2020; "Diagnosis and staging," 2024; Ning et al., 2021). The tissue samples collected are then analyzed by a pathologist in order to confirm the diagnosis and further identify the tumor's histology and assess predictive biomarkers (e.g., programmed death-ligand 1 (PD-L1) expression and molecular analysis) (Alexander et al., 2020).

When there are suspicions that the lung cancer has progressed, liquid biopsies are performed. This is a minimally invasive method that allows the monitoring of the tumor's response to the chosen treatment through the genetic analysis of circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) obtained from the patients' body fluids (e.g., blood samples) (Nooreldeen & Bach, 2021).

1.4.3. Positron Emission Tomography-Computed Tomography

Following the confirmation of lung cancer through biopsy, patients are typically submitted to a positron emission tomography-computed tomography (PET-CT) scan to evaluate metastatic disease before starting treatment ("Diagnosis and staging," 2024).

1.5. Histology

Lung cancer can be histologically subdivided in SCLC, that corresponds to 15% of all diagnosis, and non-small cell lung cancer (NSCLC), which represents 85% of all diagnosis (**Figure 1**) (Sinjab et al., 2022; Thai et al., 2021).

SCLCs originate in the central airway from pulmonary neuroendocrine cells and are strongly linked to tobacco smoking. This histosubtype grows and metastasizes rapidly, is poorly differentiated, and is often diagnosed at an advanced stage, making it the one that exhibits the poorest prognosis (Nooreldeen & Bach, 2021).

Morphologically, 40% of NSCLCs are adenocarcinomas, 25-30% are squamous cell carcinomas, and 10-15% of NSCLCs are large cell carcinomas (Schabath & Cote, 2019).

Lung adenocarcinomas (LADCs) can be further subdivided according to their morphology in lepidic, acinar, papillary, micropapillary and solid subtypes. The lepidic LADCs exhibit the best prognosis and micropapillary and solid LADCs have the worst prognosis of all morphology subtypes (Nicholson et al., 2022). LADCs are more commonly found in young patients, Asians, women and never-smokers, even though it also occurs in smokers (Saito et al., 2016).

Squamous cell carcinomas arise centrally, in the major bronchi, from basal epithelial cells (Shehata et al., 2023) and, like SCLC, are strongly associated with tobacco smoking. Although it was the most common lung cancer histosubtype some decades ago, its incidence is decreasing due to a decline in cigarette smoking rates and changes in cigarette

composition (introduction of filtered cigarettes and reduction of nicotine content). This leads to deeper inhalations, allowing the carcinogens to reach more peripheral areas of the lung, such as the alveoli, where LADCs can develop (Ruiz-Cordero & Devine, 2020).

Large cell carcinomas are undifferentiated tumors that do not present morphologic features of SCLC, adenocarcinoma (glandular differentiation) or squamous cell carcinoma (squamous differentiation) (Weissferdt, 2014).

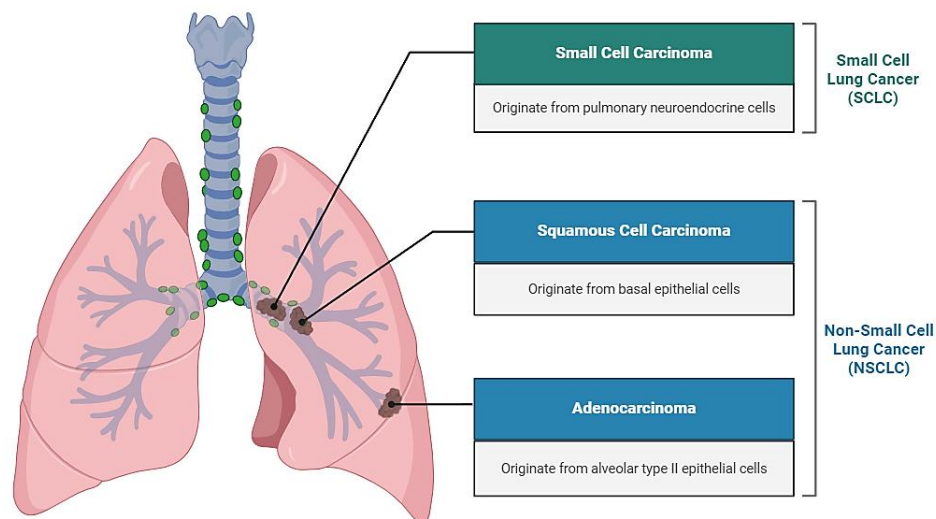


Figure 1 – Histological classification of lung cancer and cell of origin. Lung cancer can be divided into small cell lung cancer (SCLC), which arises from neuroendocrine cells, and non-small cell lung cancer (NSCLC). Within the NSCLC histology, the cancer can be further classified as adenocarcinoma, with its origin in alveolar type II cells, squamous cell carcinoma, originating from basal epithelial cells, and large cell carcinoma (not represented). The typical locations of each of these tumors within the lung are represented. Created with BioRender.com.

2. Lung Adenocarcinoma

2.1. Etiology and Development

LADC precursor lesions appear as ground glass nodules (GGNs) on CT scans. However, even though GGNs are linked to lung cancer, they can also be caused by infection, inflammation, fibrosis or hemorrhage (Pascoe et al., 2018; Succony et al., 2021). These early lesions can disappear as time goes by and they can remain the same size for several years and never progress to cancer. However, 10% of these lesions can increase in size, grow a solid component and become invasive. Additionally, GGNs are more commonly found in never-smokers, women and Asians (Succony et al., 2021).

The development of LADCs is thought to follow a continuum characterized by lesions with increasing invasiveness. This is supported by consistent morphological and molecular alterations observed across these lesions (Inamura, 2018). The continuum begins with atypical adenomatous hyperplasia (AAH) that eventually progresses to adenocarcinoma *in situ* (AIS), then to minimally invasive adenocarcinoma (MIA) and finally to invasive lung adenocarcinoma (ILA).

AAHs are preinvasive glandular lesions consisting of proliferating alveolar type II (AT2) epithelial cells presenting mild to moderate atypia (Inamura, 2018; Nicholson et al., 2022). These lesions are usually located near resected adenocarcinomas, in the small bronchi, bronchioles and alveoli of the peripheral lung (Duma et al., 2019; Sinjab et al., 2022; Wistuba & Gazdar, 2006), highlighting the role of field cancerization in lung cancer, i.e., the continuous exposure to tobacco smoking leads to the appearance of genetic alterations throughout all lung tissue (Hutchinson et al., 2019). On CT scans, AAHs appear as spherical GGNs measuring less than 5 millimeters (mm) and forming a lepidic pattern, thus lacking a solid component (Sinjab et al., 2022; Succony et al., 2021).

The AT2 cells composing an AIS exhibit more atypia than those composing AAH, creating a tumor with a diameter inferior to 3 centimeters (cm). The GGN on CT scans can appear with a lepidic morphology without a solid component, but it can also already show a part solid component (Inamura, 2018; Succony et al., 2021). This is still a preinvasive lesion, so no lymphatic, vascular, stromal and pleural invasion is observed, as well as no spread of tumor cells through the airspaces (STAS) (Succony et al., 2021).

A MIA is a predominantly lepidic lesion with a diameter of less than 3 cm and a focus of invasion of the stroma smaller than 5 mm. This invasive focus usually presents a different morphology (commonly, acinar or papillary) compared to the tumor from which it originated (Inamura, 2018). Nevertheless, if the lymphatic or circulatory system, pleura or air spaces are affected or if there is presence of tumor necrosis, the diagnosis is LADC instead of MIA (Succony et al., 2021). When fully resected, MIA is not expected to recur (Inamura, 2018), thus having a 5-year survival rate of 100% (Hutchinson et al., 2019).

An ILA exhibits an invasive focus greater than 5 mm with a morphology other than lepidic, that reaches the lymphatic and/or circulatory system, pleura and/or air spaces or that presents tumor necrosis (Succony et al., 2021).

2.2. Genetic Alterations in NSCLC

LADC is the lung cancer histologic subtype with the second highest mutational burden, following squamous cell carcinoma (Devarakonda et al., 2015). The most common mutations found in LADCs affect the Kirsten rat sarcoma virus (*KRAS*) and epidermal growth

factor receptor (*EGFR*) genes (Sucony et al., 2021). Mutations in these proto-oncogenes can initiate carcinogenesis and are therefore called driver mutations. This contrasts with passenger mutations, which do not lead to carcinogenesis (Hutchinson et al., 2019; Inamura, 2018). Mutations in *KRAS* and *EGFR* are believed to occur early in the continuum of LADC lesions (Devarakonda et al., 2015) and, since they can independently drive carcinogenesis, they are mutually exclusive (as happens with other driver mutations). Moreover, tumor cells harboring mutations in these genes rely on this mutation and on the activation of downstream signaling pathways for proliferation and survival, something that is known as “oncogene addiction” (Denisenko et al., 2018; Saito et al., 2016; Thai et al., 2021).

The frequency of mutations in these genes varies considering the smoking status of the individual, being *KRAS* indicative of smoking, and *EGFR* mutations and anaplastic lymphoma kinase (*ALK*), ROS proto-oncogene 1 (*ROS1*) and rearranged during transfection proto-oncogene (*RET*) rearrangements occurring in never-smokers (Nicholson et al., 2022; Ruiz-Cordero & Devine, 2020).

Currently, LADCs can be classified according to the driver mutations they present – molecular diagnosis – as many of these mutations have now a targeted therapy available (Denisenko et al., 2018). Therefore, current guidelines (Lindeman et al., 2018) recommend molecular testing for all newly diagnosed metastatic NSCLC patients (Thai et al., 2021). Considering this, LADCs can be molecularly classified into terminal respiratory unit (formerly bronchioid), proximal proliferative (formerly magnoid), and proximal inflammatory (formerly squamoid) subtypes. The terminal respiratory unit subtype, found predominantly in never-smoking women, harbors mutations or copy number variations (CNVs) in *EGFR* and kinase fusions. The proximal proliferative subtype is characterized by mutations and CNVs in *KRAS* and serine/threonine kinase 11 (*STK11*). The proximal inflammatory subtype presents tumor suppressor protein p53 (*TP53*) and neurofibromatosis type 1 (*NF1*) co-mutations and a solid morphology (Cancer Genome Atlas Research, 2014; Devarakonda et al., 2015; Ruiz-Cordero & Devine, 2020).

2.2.1. EGFR

EGFR, or human epidermal growth factor receptor 1 (HER1), is a tyrosine kinase receptor (RTK) that belongs to the erythroblastic leukemia viral oncogene (ERBB) family of RTKs, along with HER2, HER3 and HER4. The *EGFR* gene is composed of 28 exons that form an extracellular domain responsible for ligand binding and dimerization; a transmembrane domain; and an intracellular domain, that includes the tyrosine kinase domain divided into a N-lobe and a C-lobe, with an adenosine triphosphate (ATP) binding

site in-between (Ruiz-Cordero & Devine, 2020; Wee & Wang, 2017). When an EGFR ligand, such as the epidermal growth factor (EGF), binds to EGFR, the receptor suffers a conformational change and homo- or heterodimerizes with other ERBB family members. When the intracellular N-lobe of one monomer interacts with the C-lobe of the other monomer, trans-autophosphorylation of the intracellular tyrosine kinase domains occurs (Wee & Wang, 2017) and three main signaling pathways are then activated: Raf-MEK-ERK (rapidly accelerated fibrosarcoma – mitogen-activated protein kinase kinase – extracellular signal-regulated kinase), PI3K-AKT (phosphatidylinositol-3-kinase – protein kinase B) and JAK-STAT (Janus kinase – signal transducers and activators of transcription) (Denisenko et al., 2018; Ruiz-Cordero & Devine, 2020).

Mutations in the *EGFR* gene affect 10-16% of European LADC patients and 62% of Asian patients (Thai et al., 2021). *EGFR* mutations mainly occur at mutational hotspots, and, in the case of NSCLC, this coincides with exons 18 to 21, which encode the ATP binding site of the tyrosine kinase domain (exons 18 to 24) of *EGFR*. Particularly, the most common mutations are exon 19 in-frame deletions and the L858R point mutation in exon 21 (substitution of a leucine (L) for an arginine (R) in codon 858). These mutations lead to the ligand-independent constitutive activation of EGFR, causing uncontrollable cell proliferation, survival and inhibition of apoptosis (Ruiz-Cordero & Devine, 2020).

2.2.2. Other Driver Mutations

Other NSCLC driver mutations currently tested in molecular diagnosis include the following:

Mutations occurring in the *KRAS* gene are the most frequently encountered in LADC patients, impacting up to 30% of NSCLC patients. Despite generally affecting codons 12 and 13, the most common *KRAS* mutation is the aminoacidic substitution of a glycine with a cytosine in codon 12 (G12C). This then leads to the constitutive activation of the *KRAS* protein, due to its impaired GTPase activity (Denisenko et al., 2018; Devarakonda et al., 2015).

Driver mutations in v-raf murine sarcoma viral oncogene homologue B1 (*BRAF*), mainly missense mutations that exchange a valine for another aminoacid in codon 600 (V600), are also responsible for driving 5-8% of LADC carcinogenesis, since the mutation results in the constitutive activation of *BRAF* (Devarakonda et al., 2015).

MET proto-oncogene receptor tyrosine kinase (*MET*) mutations, which can be amplifications or exon 14 skipping mutations (consequence of splice site mutations causing the loss of a *MET* negative regulatory site (Devarakonda et al., 2015)), occur in less than 5% of LADC patients (Chevallier et al., 2021).

ALK rearrangements can result from the fusion of *ALK* and another gene, usually echinoderm microtubule-associated protein-like 4 (*EML4*), due to a paracentric inversion on chromosome 2p (Saito et al., 2016). This results in an active and upregulated *ALK* that does not physiologically exist in the lung tissue. These *ALK* fusions are found in 3-8% of LADC patients (Devarakonda et al., 2015).

ERBB2 mutations, occurring in 1-3% of LADC patients, are usually amplifications or exon 20 insertions (Devarakonda et al., 2015).

RET rearrangements frequently consist in an inversion on chromosome 10, creating a fusion between this gene and the kinesin family member 5B (*KIF5B*) or coiled-coil domain containing 6 (*CCDC6*) genes. *RET* fusions occur in 1-2% of LADC patients (Chevallier et al., 2021).

ROS1 forms fusions with over 20 different partner genes in NSCLC. Nevertheless, the most common ones involve cluster of differentiation 74 (*CD74*), ezrin (*EZR*), syndecan 4 (*SDC4*) and solute carrier family 34 member 2 (*SLC34A2*). These *ROS1* fusions occur in 1-2% of LADC patients (Chevallier et al., 2021).

Neurotrophic tyrosine receptor kinase (*NTRK*) gene rearrangements with more than 25 fusion partners occur in 1% of LADC patients (Chevallier et al., 2021).

Gene fusions involving the neuregulin-1 (*NRG1*) gene occur in 0.3% of NSCLC cases. The most common fusion partner is *CD74* (Jonna et al., 2019).

Still, around 30-50% of LADCs patients lack the driver mutations listed above (Denisenko et al., 2018; Saito et al., 2016).

3. NSCLC Treatment

The treatment for lung cancer varies and it is based on the patients' condition and their cancer's histology, stage and genetic alterations (Alexander et al., 2020). Clinicians follow standard treatment options recommended by the European Society for Medical Oncology (ESMO) according to the lung cancer stage: early stage (stages I and II), locally advanced (stage III) and metastatic (stage IV) NSCLC.

3.1. Early Stage NSCLC

3.1.1. Surgery

When lung cancer is diagnosed at an early resectable stage, the treatment has a curative intent and involves surgery to remove the tumor. This can be done through

thoracotomy (open chest surgery) or video-assisted thoracoscopic surgery (VATS) (Thai et al., 2021).

Different types of surgeries can be performed, depending on the size and anatomical location of the tumor and depending on the patients' condition. However, lobectomy, which involves extracting a single lobe of the lung (Duma et al., 2019), is the standard of care for patients with early stage lung cancer. Nevertheless, for patients who cannot tolerate lobectomy due to poor pulmonary function, sublobar resection is performed to preserve lung function (Hutchinson et al., 2019; Thai et al., 2021).

For stage II patients, adjuvant chemotherapy is recommended after resection of the tumor (Alexander et al., 2020; Duma et al., 2019). Moreover, for patients with incomplete resection, radiation therapy is recommended (Nasim et al., 2019).

3.1.2. Radiotherapy

In the case of patients with an early stage NSCLC who cannot (or do not want to) undergo surgery, stereotactic ablative body radiotherapy (SABR) can be considered over conventional radiotherapy (Duma et al., 2019; Nasim et al., 2019) since it improves survival (Thai et al., 2021). SABR involves delivering high doses of radiation to the tumor in one to five fractions while minimizing exposure to the surrounding organs (Chang, 2015).

3.2. Locally Advanced NSCLC

If the locally advanced NSCLC is resectable, patients can receive chemotherapy or chemoradiotherapy (CRT) as induction therapy (i.e., treatment performed in order to decrease tumor size) followed by surgery or they can receive chemotherapy as adjuvant therapy after surgery. On the other hand, if the locally advanced lung tumor is unresectable, concurrent CRT is given. This can be followed by the immunotherapy drug durvalumab (PD-L1 inhibitor) if the PD-L1 expression is $\geq 1\%$ on tumor cells and if there is no tumor progression after CRT (*Lung & Chest Cancers: Pocket Guideline*, 2023).

3.3. Metastatic NSCLC

Initially, lung cancer metastasizes to the lymph nodes, after which it can spread to other organs, typically the brain, liver, bones, and adrenal glands (Popper, 2016).

Molecular testing of LADCs in search for the driver mutations described above is recommended since they can now be treated with targeted therapies. Additionally, testing the PD-L1 expression by tumor cells is also recommended for NSCLC treatment with immunotherapies (Nasim et al., 2019; Ruiz-Cordero & Devine, 2020).

3.3.1. Immunotherapy (Monotherapy or Combination)

Programmed death-1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4) are immune checkpoints expressed on the surface of immune cells (e.g., T lymphocytes) that when interact with their ligands, PD-L1 and cluster of differentiation 80/86 (CD80/86) respectively, prevent immune activation against self-antigens (Lao et al., 2022; Thai et al., 2021).

In cancer, tumor cells have an upregulated expression of PD-L1 and CD80/86 thereby escaping immune recognition by inhibiting the activation of PD-1- and CTLA-4-expressing immune cells. Nevertheless, almost a decade ago, immune checkpoint inhibitors (ICIs), namely anti-CTLA4, anti-PD-1 and anti-PD-L1 drugs, started being used for lung cancer treatment. These drugs block the inhibitory signals exchanged between tumor and immune cells and consequently allow the identification and elimination of tumor cells (Lao et al., 2022). For this reason, it is crucial that newly diagnosed patients are tested for PD-L1 expression (Thai et al., 2021).

Immunotherapy drugs function as first-line treatment for NSCLC patients who lack mutations that confer sensitivity to targeted therapies. These drugs can be given either as monotherapy or in combination with other immunotherapies or with chemotherapy.

When the tumor has a PD-L1 expression greater than or equal to 50%, the anti-PD-1 inhibitor pembrolizumab is the standard treatment. Moreover, the anti-PD-L1 drug atezolizumab is approved for treatment when the tumor has a PD-L1 expression greater than or equal to 50% and when the tumor infiltrating immune cells have a PD-L1 expression greater than or equal to 10% (Alexander et al., 2020; Thai et al., 2021).

The combination of the anti-PD-1 drug nivolumab with the anti-CTLA-4 drug ipilimumab has been Food and Drug Administration (FDA) approved for the treatment of NSCLC with PD-L1 expression greater than or equal to 1% (Alexander et al., 2020; Thai et al., 2021).

Several clinical trials have shown that the combination of immunotherapy with chemotherapy improved progression-free survival (PFS) and overall survival (OS) of NSCLC patients. In fact, some of these chemoimmunotherapy combinations are already approved as first-line treatment for NSCLC by the FDA and European Medicines Agency (EMA): pembrolizumab plus chemotherapy; atezolizumab plus bevacizumab, carboplatin and paclitaxel (ABCP) for non-squamous NSCLC; and atezolizumab plus carboplatin and nanoparticle albumin-bound (nab)-paclitaxel for non-squamous NSCLC (Thai et al., 2021).

When metastatic NSCLC patients have contraindications for immunotherapy, platinum-based doublet chemotherapy is the preferred treatment (*Lung & Chest Cancers: Pocket Guideline*, 2023).

3.3.2. Targeted Therapy for EGFR^{mut+} LADC

As previously said, mutations in *EGFR* frequently affect its tyrosine kinase domain, causing constitutive activation of this RTK and consequent proliferation of tumor cells. Therefore, at the beginning of the 21st century, tyrosine kinase inhibitors (TKIs) began to emerge and, some years later, became the first-line treatment for metastatic LADC patients with sensitizing mutations in *EGFR*. This change occurred because TKIs show an improved OS and PFS rates and decreased adverse effects when compared to the former standard platinum-doublet chemotherapy (carboplatin or cisplatin + gemcitabine, vinorelbine, or taxanes) (Duma et al., 2019).

EGFR mutations in LADC exhibit different sensitivities to TKIs. Specifically, exon 19 deletions and the exon 21 L858R point mutation confer sensitivity to TKIs, whereas insertions in exon 20 typically confer resistance to most of these drugs and so are treated with the standard platinum-doublet chemotherapy (Thai et al., 2021).

There are three generations of EGFR TKIs approved by EMA and FDA for the treatment of *EGFR* mutated LADCs. The mechanism of action of these drugs is similar across all generations and consists in binding to the ATP binding pocket, located in the tyrosine kinase domain of EGFR, and preventing ATP from interacting with it. This blocks the downstream signaling pathways that would promote the production of cyclin D1, that forms a complex with the cyclin-dependent kinases 4 and 6 (CDK4/6) and promotes the progression of the cell cycle from the G1 phase to the S phase (**Figure 2**), and the downregulation of CDK inhibitor proteins, namely the p27^{KIP1} protein that inhibits the cyclin D1-CDK4/6 complex (Wee & Wang, 2017). The first-generation of EGFR TKIs includes erlotinib and gefitinib, which bind reversibly to the ATP binding site in EGFR. Afatinib and dacomitinib, pan-HER inhibitors (Hendriks et al., 2023), belong to the second-generation of TKIs, binding irreversibly to the same site in EGFR. Finally, osimertinib, a third-generation drug, targets both *EGFR* sensitizing mutations and the *EGFR* mutation that most commonly confers resistance to first-generation TKIs in tumor progression: the aminoacidic substitution of a threonine to a methionine in codon 790 (T790M) in exon 20. Osimertinib functions as a selective ATP competitive inhibitor by covalently binding to the cysteine residue in codon 797 in the ATP binding site of EGFR and decreasing the EGFR affinity for ATP caused by the T790M mutation (Ruiz-Cordero & Devine, 2020). Additionally, osimertinib shows better control of central nervous system (CNS) metastasis, which,

together with the fact that it targets resistance mutations, made this drug the first-line TKI (Thai et al., 2021).

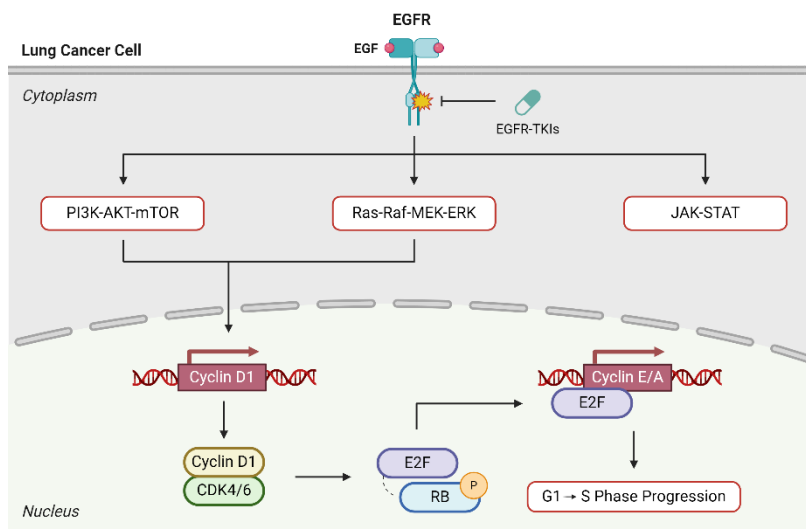


Figure 2 – Mutations in *EGFR* guide the upregulation of genes that promote cell cycle progression. The EGFR downstream signaling pathways PI3K-AKT and Raf-MEK-ERK lead to the transcription of the gene encoding cyclin D1 (*CCND1*), which forms a complex with CDK4/6. This complex phosphorylates retinoblastoma (RB) and releases the E2F transcription factor (E2F) from the RB-E2F complex. E2F, in turn, will help the transcription of cyclin E and cyclin A, that promote the progression of the cell cycle from the G1 phase to the S phase, thus promoting the proliferation of lung cancer cells. EGFR-TKIs inhibit the phosphorylation and activation of EGFR, thereby blocking these downstream signaling pathways and the proliferation of cancer cells. Created with BioRender.com.

3.3.3. Targeted Therapy for Other LADC Driver Mutations

At present, NSCLC patients with a G12C *KRAS* mutation follow the treatment options available for metastatic NSCLC without driver mutations. Hence, these patients are treated with immune checkpoint inhibitors in monotherapy. If this therapy fails, sotorasib or adagrasib (not EMA approved), drugs that bind to the mutation site and lock *KRAS* in its inactive guanosine diphosphate (GDP)-bound state (Thai et al., 2021), can be administered (Hendriks et al., 2023).

For V600 mutations in *BRAF*, the BRAF-MEK inhibitors, dabrafenib and trametinib respectively, are used as first-line treatment (*Lung & Chest Cancers: Pocket Guideline*, 2023).

For lung tumors with an exon 14 skipping mutation on *MET* gene, FDA approved the use of the selective MET inhibitors capmatinib and tepotinib as first- or second-line therapy (Chevallier et al., 2021; *Lung & Chest Cancers: Pocket Guideline*, 2023).

Currently, there are three generations of ALK TKIs: crizotinib belongs to the first-generation; ceritinib, alectinib and brigatinib to the second-generation; and lorlatinib to the third-generation of ALK TKIs (this drug has activity against most ALK TKI resistance

mutations). Patients with LADCs that exhibit *ALK* fusions are treated with alectinib, brigatinib or lorlatinib as first-line (*Lung & Chest Cancers: Pocket Guideline*, 2023).

Lung cancer patients that present exon 20 insertions in *ERBB2* gene can be treated first-line with the antibody-drug conjugate trastuzumab deruxtecan, as approved by the FDA (*Lung & Chest Cancers: Pocket Guideline*, 2023).

The recommended first-line treatments for *RET* rearrangements are the selective RET inhibitors selpercatinib or pralsetinib (Chevallier et al., 2021; *Lung & Chest Cancers: Pocket Guideline*, 2023).

Patients with *ROS1* fusions are treated with crizotinib or entrectinib, being the latter preferred if brain metastasis are present (*Lung & Chest Cancers: Pocket Guideline*, 2023).

NTRK rearrangements can be treated with the NRTK inhibitors larotrectinib and entrectinib (*Lung & Chest Cancers: Pocket Guideline*, 2023).

Lastly, lung cancers with *NRG1* fusions currently do not have an approved treatment, but HER inhibitors, such as afatinib, have shown promising results in clinical trials (Chevallier et al., 2021).

3.3.4. EGFR TKI Therapy Resistance

As mentioned previously, liquid biopsies are performed on patients when there are suspicions that the lung cancer has progressed. The ctDNA in the plasma is analyzed using a polymerase chain reaction (PCR) test or next-generation sequencing (NGS) in order to detect mutations in the *EGFR* gene which may be responsible for the resistance to the current treatment (Thai et al., 2021).

Therapy resistance can be primary/innate or secondary/acquired. Primary resistance occurs when pre-existing genetic alterations confer resistance to certain treatments before they are administered. Secondary resistance emerges when a tumor acquires resistance under the selective pressure of an initial treatment, either due to a subclone that becomes predominant in the tumor or due to new resistance mutations arising (Chatterjee & Bivona, 2019). The initial therapy eliminates the tumor cells sensitive to the treatment, however, those with resistance mutations survive, grow and proliferate, eventually becoming the predominant clones in the tumor mass. There are two main types of acquired resistance: on-target and off-target. On-target resistance occurs when additional alterations in the target gene (in this case, *EGFR*) prevent the drug from binding effectively. These can be second-site mutations, gatekeeper mutations (e.g., T790M mutation), solvent-front mutations (e.g., G796S/R mutation, which alter the osimertinib binding affinity via steric hindrance), compound mutations, and *EGFR* amplification. Off-target resistance happens when signaling pathways downstream of the drug's target are re-activated by being

bypassed (e.g., mutations in *BRAF* and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*)) or when there is activation of parallel signaling pathways downstream of other RTKs that lead to proliferation (e.g., *MET* and *ERBB2* amplifications). Finally, additional EGFR TKI resistance mechanisms can occur, such as phenotypic transformation, which consists in an alteration of the tumor's histology (e.g., NSCLC transforming into SCLC) (Rotow & Bivona, 2017; Thai et al., 2021).

As previously noted, in 50-60% of patients that progress on first- and second-generation EGFR TKIs, the resistance mutation identified is the T790M in exon 20 of *EGFR* (Hendriks et al., 2023). The change of a threonine for a bulky methionine causes the ATP binding pocket of EGFR to get an increased affinity for ATP and a lower affinity for EGFR TKIs (Bronte et al., 2014). Resistance to first and second-generation EGFR TKIs typically develops 6-12 months after this initial treatment (Duma et al., 2019), and, then, patients can be treated with osimertinib. Nevertheless, resistance is also imminent with osimertinib therapy, occurring due to the exchange of a cysteine for a serine in codon 797 (C797S) in exon 20 of *EGFR*. If the T790M and C797S resistance mutations occur in separate *EGFR* alleles (in trans), the tumor is sensitive to a combination of first and third-generation EGFR TKIs, but not to osimertinib in monotherapy. On the other hand, if the T790M and C797S resistance mutations occur in the same *EGFR* allele (in cis), the tumor becomes resistant to all generations of EGFR TKIs (Niederst et al., 2015). When this happens, the standard treatment shifts to platinum-doublet chemotherapy (Hendriks et al., 2023). Hence, therapy resistance is a major issue in the management of lung cancer.

4. Preliminary Results and Research Hypothesis

Through the examination of liquid and tissue biopsies of LADC patients after tumor progression on erlotinib treatment, our group found that, in patients with relapsing tumors, the allelic frequency of erlotinib sensitizing mutations is on average 3.2-fold higher than the allelic frequency of the p.T790M resistance mutation (**Figure 3**) (Junqueira-Neto et al., 2022), showcasing that, within the relapsing tumor cells, only a subpopulation of the resistant cancer cells carries the therapy resistance mutation despite the common assessment that relapsing tumors are solely comprised of a predominant resistant clone selected by therapy pressure.

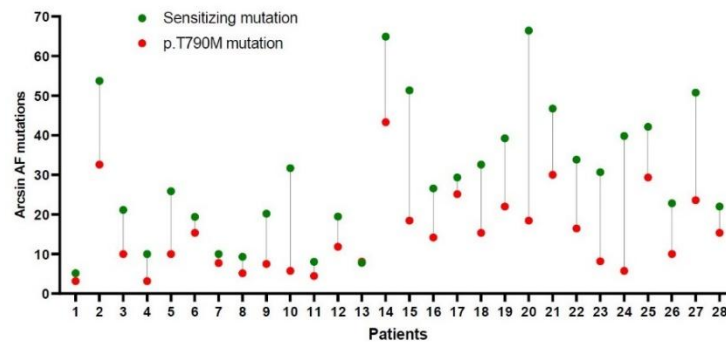


Figure 3 – The allelic frequency (AF) of erlotinib sensitizing mutations is higher than the AF of the p.T790M erlotinib-resistant mutation in relapsing LADCs. Liquid biopsies from 14 patients and tissue biopsies from additional 14 patients were analyzed by NGS after tumor progression. The relapsing tumors showed, on average, a 3.2-fold higher AF of the initial sensitizing mutation (represented by the green dots) than of the resistance mutation (represented by the red dots). Unpublished data.

The development of resistance was also assessed by performing *in vitro* and *in vivo* assays. The *in vitro* experiment used the HCC827 LADC erlotinib-sensitive cell line, that harbors the E746-A750 deletion in exon 19 of the *EGFR* gene, and the H1975 LADC erlotinib-resistant cell line, that harbors both the erlotinib-sensitive p.L858R mutation in exon 21 and the erlotinib-resistant p.T790M mutation in exon 20 of the *EGFR* gene, making these cells erlotinib-resistant. The experiment involved comparing the time to acquire resistance of HCC827 cells supplemented with conditioned media (CM) from H1975 cells and treated with erlotinib with HCC827 cells only treated with erlotinib (**Figure 4**). Initially, HCC827 cells responded to the erlotinib therapy, but soon developed resistance to this treatment, even when the supplementation with CM was withdrawn and the erlotinib maintained.

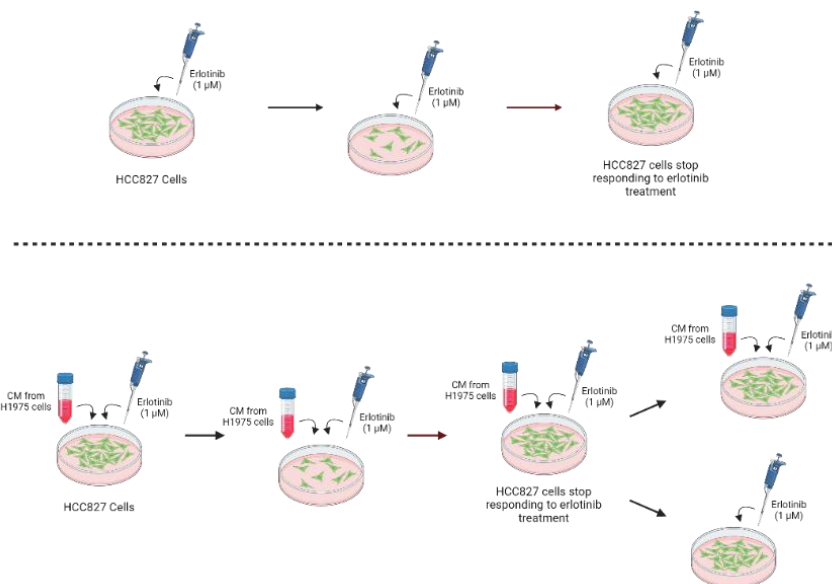


Figure 4 – Experimental outline of cell culture conditions using erlotinib-sensitive HCC827 cells and CM from erlotinib-resistant H1975 cells. In the upper panel, HCC827 cells were treated with erlotinib and acquired resistance to this treatment through habituation. In the bottom panel, on the other hand, HCC827 cells were supplemented with CM from H1975 cells and acquired a CM-derived transferred resistance to erlotinib. Then, the CM stimulus was removed and the treatment with erlotinib maintained, and the cells were still resistant to the EGFR-TKI therapy. Adapted from (Junqueira-Neto et al., 2022).

The *in vivo* experiment used C57BL/6 Rag2^{-/-}; IL2rg^{-/-} immunodeficient mice that were either single inoculated with HCC827 cells or dual inoculated with HCC827 and H1975 cells. Erlotinib treatment followed the inoculation of these LADC cell lines (**Figure 5**).

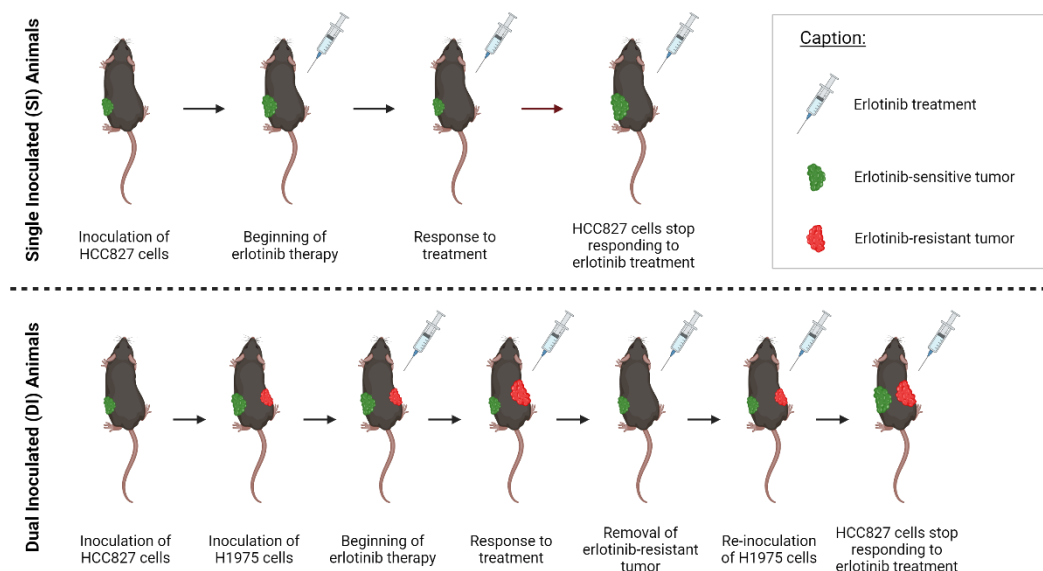


Figure 5 – C57BL/6 Rag2^{-/-}; IL2rg^{-/-} immunodeficient mice were inoculated with HCC827 and H1975 cells. One mouse model was single inoculated with HCC827 cells and, when the tumor reached 1000 mm³, erlotinib was administered by oral gavage. Another mouse model was dual inoculated, in opposite flanks, with HCC827 and H1975 cells and further treated with erlotinib. Eventually, both sensitive tumors stopped responding to the erlotinib treatment. Adapted from (Junqueira-Neto et al., 2022).

The cells supplemented with CM and the dual inoculated mice acquired resistance to erlotinib treatment faster than HCC827 cells treated only with erlotinib and the single inoculated mice, respectively. Additionally, the common p.T790M resistance mutation was not detected in the former and these results were consistent with what was found in human patients. Moreover, it was confirmed that the resistance was not being caused by other mechanisms, such as mutations in *EGFR*, *KRAS*, neuroblastoma rat sarcoma viral oncogene homolog (*NRAS*), *BRAF* and *PIK3CA* and CNVs in *ERBB2*, *MET* and *BRAF* and, consequently, these tumors were classified as having a resistance phenotype (Junqueira-Neto et al., 2022).

To further investigate this, tumors comprised of sensitive cells before and after the acquisition of resistance were sequenced. The ribonucleic acid (RNA)-seq results were then analyzed through the “Gene Ontology” and the “KEGG pathway” platforms. The results obtained showed a significant upregulation of genes associated with the endocytosis pathway in tumors with the resistance phenotype (Erlotinib-Resistant S (ERS) tumors) when compared to erlotinib-sensitive tumors (Erlotinib-Sensitive S (ESS) tumors) (**Figure 6A**). Within this pathway, the most significantly overexpressed genes were the ones that encode caveolins-1 (*CAV1*) and -2 (*CAV2*) (**Figure 6B**) (Junqueira-Neto et al., 2022).

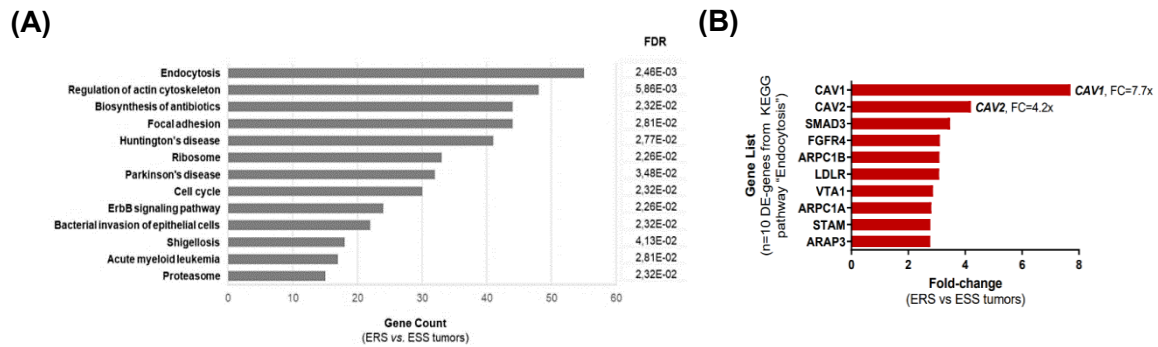


Figure 6 – Genes associated with the endocytosis pathway, namely *CAV1* and *CAV2*, were significantly upregulated in tumors exhibiting the resistance phenotype (ERS tumors) when compared to tumors sensitive to the erlotinib treatment (ESS tumors). (A) The “Gene Ontology” and the “KEGG pathway” platforms identified 55 endocytosis genes that were more expressed in ERS than in ESS tumors. **(B)** *CAV1* and *CAV2* were the genes with the highest fold change (FC) between ERS and ESS tumors (Junqueira-Neto et al., 2022). FDR = False Discovery Rate.

Caveolins are integral membrane proteins that are an essential structural component for the formation of caveolae (plasma membrane invaginations) together with cavins. There are three protein isoforms in the caveolin family: *CAV1* and *CAV2* are often co-expressed and found predominantly in epithelial cells, endothelial cells and adipocytes, and *CAV3* is mainly expressed in striated and smooth muscle cells (Fridolfsson et al., 2014). These proteins can form homo- or hetero-oligomers with each other and interact with homo- or hetero-trimers of cavins to stabilize the caveolar structure. Caveolae, in turn, play a crucial role in endocytosis, and signal transduction by selectively concentrate proteins and facilitate interactions between them (Dudau et al., 2020).

Considering these preliminary results, a research hypothesis emerged based on the knowledge of horizontal gene transfer (HGT), which is the transfer of genetic material between prokaryotes and eukaryotes. In prokaryotic organisms, HGT occurs mainly by transformation, transduction, and conjugation, which, for instance, allow bacteria to acquire antibiotic resistance genes, thereby enhancing their adaptive capabilities. On the other hand, in eukaryotic organisms, HGT occurs through circulating cell-free DNA (cfDNA), apoptotic bodies and extracellular vesicles (EVs) (Emamalipour et al., 2020).

Given that the rapid accumulation of erlotinib-resistant cells cannot be explained solely by mitosis-driven vertical transmission of resistance, the hypothesis of horizontal transmission of resistance emerged, suggesting that the transmission of resistance between mutated and non-mutated lung cancer cells happens through a mutation-independent mechanism via the secretome or via the release of molecules encapsulated in EVs that can alter the phenotype of the recipient cells into a resistant one (**Figure 7**).

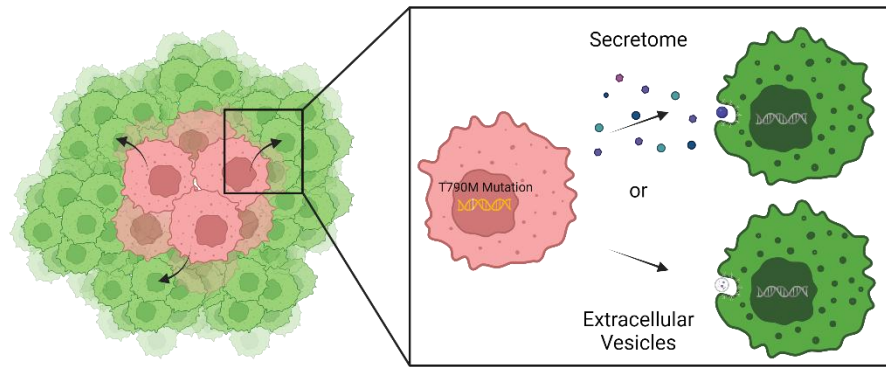


Figure 7 – The hypothesis of horizontal transmission of resistance. Created with BioRender.com.

Moreover, our group proved that the acquired erlotinib resistance is a stable phenotype that is transmitted to the daughter tumor cells without the triggering of any common genetic alterations. This resistance was maintained *in vitro*, when the CM from H1975 was removed from HCC827 cells and these were just treated with erlotinib (**Figure 4**), and *in vivo*, when tumors comprised of sensitive cells that acquired the resistance phenotype remained resistant to erlotinib after being transplanted to new mice (**Figure 8**). As mentioned previously, *in vitro* and *in vivo* digital PCR results further confirmed the absence of the p.T790M resistance mutation, reinforcing that the acquisition of resistance to erlotinib treatment did not occur through cell migration nor through the transference of mutated DNA and, therefore, is not dependent on the acquisition of the resistance mutation (Junqueira-Neto et al., 2022). Hence, our group hypothesized that molecules secreted from erlotinib-resistant cells may promote the acquisition of epigenetic modifications in erlotinib-sensitive cells, leading to the stable resistance phenotype exhibited.

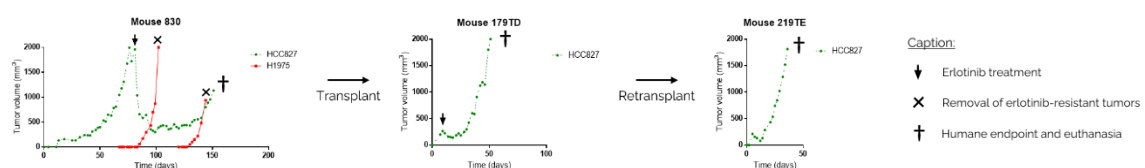


Figure 8 – The erlotinib resistance phenotype is a stable characteristic of HCC827 tumors removed from dual inoculated animals. On the graph on the left, it is possible to see that erlotinib-sensitive tumors, comprised of HCC827 cells (green line), develop resistance faster when in the presence of erlotinib-resistant tumors, comprised of H1975 cells (red lines), establishing tumors with the resistance phenotype mentioned. When these tumors that display the resistance phenotype are transplanted into new mice and then treated with erlotinib (middle and right graphs), they continue to exhibit resistance, even in the absence of the H1975 cells' stimulus (Junqueira-Neto et al., 2022).

Additionally, results from a phospho-kinase array, using *in vivo* tumor samples before and after the acquisition of the resistance phenotype, showed that some proteins and kinases downstream of caveolin exhibited significantly different phosphorylation levels, denoting their increased activation, between erlotinib-sensitive tumors and tumors that

acquired the resistance phenotype (**Table 1**). Considering that some of these proteins are known to be downstream of EGFR (e.g., ERK and AKT), our group suspected of an off-target resistance mechanism and hypothesized that inhibiting these downstream molecules might resensitize the tumor cells for erlotinib treatment.

Table 1 – Statistically significant (p-value < 0.05) phospho-kinase array results. The proteins listed exhibited significantly different phosphorylation levels when comparing ERS and ESS tumors, showing a greater phosphorylation in ERS tumors than in ESS tumors. The phosphorylation site of each protein is represented. GSK-3A/B = Glycogen Synthase Kinase 3 Alpha/Beta. RSK1/2 = Ribosomal S6 Kinase 1/2. HSP60 = Heat Shock Protein 60. MSK1/2 = Mitogen- and Stress-activated protein Kinase 1/2. Unpublished data.

Targets	Phosphorylation Site	P-Value
GSK-3A	S21	0.020269
RSK1/2	S221/S227	0.027161
GSK-3B	S9	0.031159
STAT1	Y701	0.032587
ERK1/2	T202/Y204, T185/Y187	0.032739
HSP60	-	0.036636
MSK1/2	S376/S360	0.043229
AKT1	T308	0.043490

Study Aims

Considering the background described, the overarching goal of this project, conducted as part of my Master's Degree in Oncology, was to explore how the overexpression of CAV1 contributes to the development of resistance to EGFR-TKI therapies in LADC. This study sought to achieve the following specific objectives:

- **Objective 1:** Assess whether chemical inhibitors targeting molecules downstream of CAV1 can restore sensitivity to erlotinib treatment in HCC827 cells that display the resistant phenotype.
- **Objective 2:** Compare DNA methylation patterns in HCC827 cells before and after they acquire resistance to erlotinib, providing insights into epigenetic changes associated with resistance.
- **Objective 3:** Validate the role of CAV1 in promoting resistance to erlotinib by using a knockdown model to determine its contribution to the resistance phenotype.

Materials and Methods

1. Cell Lines and Experimental Conditions

The HCC827 adherent cell line (ATCC Cat# CRL-2868™), first isolated from the lungs of a 39 year old white female with LADC and established in 1994 by the depositors Adi F. Gazdar and John D. Minna (*Product Sheet: HCC827*), carries an E746-A750 deletion in exon 19 of the *EGFR* gene (Belloni et al., 2024), which confers sensitivity to therapy with EGFR TKIs.

The H1975 adherent cell line (ATCC Cat# CRL-5908™), isolated from the lungs of a never-smoker female with LADC and established in 1988 by the depositors Adi F. Gazdar and John D. Minna (*Product Sheet: NCI-H1975*), carries the p.L858R erlotinib-sensitive mutation and the p.T790M erlotinib-resistant mutation in exons 21 and 20, respectively, of the *EGFR* gene, making these cells erlotinib-resistant (*Lung Cancer Panel*, 2022).

HCC827 cells supplemented with CM from H1975 cells and treated with 1 μ M of erlotinib were also kept in culture. The erlotinib stock solution (LC Laboratories Cat# E-4007) was diluted in a solution composed of 0.5% (wt/vol) methylcellulose (Sigma-Aldrich Cat# M7027-100G) (prepared with type II H₂O) and 0.4% (vol/vol) of tween-80 (Sigma-Aldrich Cat# P4780-100ML).

The cell lines were maintained in culture using the RPMI-1640 culture medium (VWR Cat# 392-0427P) supplemented with 10% fetal bovine serum (FBS) (Cytiva Cat# SV30160.03HI) and a mixture of 100 U/mL of penicillin and 100 μ g/mL of streptomycin (Alfagene Cat# 15140122). The cells were stored in a 37°C, 5% CO₂ incubator (Galaxy 170 R, New Brunswick).

2. Mouse Models

Tumor cells were inoculated in 8-12 weeks old C57BL/6 *Rag2*^{-/-}; *IL2rg*^{-/-} immunodeficient mice under two experimental conditions.

The mice were either single inoculated with 5×10^6 HCC827 cells by subcutaneous injection into their dorsal flank, resulting in the formation of ESS tumors, or dual inoculated on opposite flanks with 5×10^6 HCC827 cells and 1×10^5 H1975 cells (when the HCC827 cells grew enough to originate a tumor with $\approx 800 \text{ mm}^3$), leading to the development of Resistant (R) and ERS tumors.

For both conditions, when the erlotinib-sensitive tumors reached $\approx 1000 \text{ mm}^3$, 25 mg/kg of erlotinib was administered by oral gavage, 3 times a week. When mice reached

the defined humane endpoints, euthanasia was performed and their tumors were dissected and formalin-fixed, paraffin-embedded (FFPE).

The mice were kept in a conventional animal holding room in the Animal Facility of Instituto de Investigação e Inovação em Saúde (i3S) with a temperature of 20-24°C, a humidity of 45-65% and a regular 12h light/12h dark cycle. The cage was provided with environmental enrichment supplies, food in the form of pellets and water *ad libitum*. All procedures followed the guidelines of the Federation of Laboratory Animal Sciences Associations (FELASA).

3. IC50 Measurement

24- and 48-hour IC50 (inhibitory concentration) assays were performed on HCC827 and H1975 parental cell lines and on HCC827 cells that exhibit the resistance phenotype to determine the drug's concentration to be used. The drugs used are small molecular inhibitors of proteins downstream of caveolin, namely the AKT inhibitor miransertib (MedChem Express Cat# HY-19719), the PI3K inhibitor wortmannin (MedChem Express Cat# HY-10197), and the ERK inhibitor SCH772984 (MedChem Express Cat# HY-50846). Miransertib was tested at concentrations ranging from 1 µM to 60 µM, wortmannin from 1 µM to 120 µM, and SCH772984 from 1 µM to 30 µM. The effect of the drugs' solvent dimethyl sulfoxide (DMSO) (Santa Cruz Biotechnology Cat# sc-202581) on cell viability was also analyzed.

4. MTT Assays

Small molecular inhibitors of proteins downstream of caveolin, namely miransertib, wortmannin, and SCH772984, were used in combination with erlotinib to assess the potential reduction of resistance to erlotinib.

The MTT assays performed on HCC827 parental cells and on HCC827 cells that exhibit the resistance phenotype had an incubation period of 48 hours with the drug and erlotinib, considering previous IC50 assays.

The MTT assays were carried out in 96-well plates and the following experimental conditions existed: negative control, control for the drug or DMSO, control for methylcellulose, 0.1 µM of erlotinib and 1 µM of erlotinib.

On the first day, 1×10^4 HCC827 parental cells and HCC827 cells supplemented with CM were plated in two separate 96-well plates and incubated for 24 hours at 37°C with

5% CO₂. In the next day, the cells were incubated with either the drug (its chosen concentration based on the IC₅₀ assays) or its vehicle, DMSO. On the third day, in addition to being supplemented with either the drug or DMSO, the cells were also supplemented with methylcellulose (erlotinib's solvent) and erlotinib (0.1 and 1 µM) and incubated for 48 hours. On the last day, 10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich Cat# M5655-500MG) was added to each well. After three hours of incubation at 37°C in the dark, the solutions in the wells were discarded and, in a flow hood, DMSO was added to the wells to solubilize the formazan crystals formed. Finally, the absorbances were read at 560 nm using a microplate reader (SynergyMx, BioTek).

5. DNA Extraction and Quantification

DNA samples of erlotinib-sensitive HCC827 cells and of HCC827 cells that display the resistance phenotype (technical replicates) were analyzed using a DNA methylation array. For this, DNA extraction, using the DNeasy Blood & Tissue Kit (Qiagen Cat# 69504), and quantification, using the Qubit fluorometer (Invitrogen Cat# Q32866), was performed. All cells were incubated with trypsin-ethylenediamine tetra-acetic acid (EDTA) 1x solution (Alfagene Cat# 15400054) for the appropriate amount of time and the resulting cell suspension was collected to a 50 mL falcon and centrifuged at 300 g for 5 minutes. Then, the supernatant was discarded, and the pellet resuspended in phosphate buffered saline (PBS) and proteinase K. The solution was transferred to an eppendorf where the lysis buffer AL was added. Then, the cells were incubated for 10 minutes, at 56°C. Following this period, ethanol was added to the eppendorf and, then, the whole mixture was transferred to a DNeasy Mini spin column placed inside a collection tube. From this moment on, a series of centrifugations and buffers were employed to complete the DNA extraction process. First, the tube was centrifuged at 6000 g for 1 minute and the flow-through discarded. The spin column was placed inside a new collection tube and the wash buffer AW1 was added. A new centrifugation at 6000 g for 1 minute was performed. Again, the flow-through was discarded and the spin column placed inside a new collection tube. This time, the wash buffer AW2 was added, and the tube was centrifuged at 20000 g for 3 minutes. The flow-through was discarded and the elution buffer AE was added to the spin column which was transferred to an eppendorf. After an incubation at room temperature (15-25°C) for 1 minute, a new centrifugation at 6000 g for 1 minute occurred, and the DNA extraction protocol was completed.

Afterwards, DNA quantification was performed by initially mixing the DNA sample with a fluorophore mix (Qubit dsDNA broad range buffer + fluorophore dsDNA broad range

reagent) in Qubit assay tubes. These tubes were incubated in the dark, at room temperature, for 2 minutes, and then the amount of DNA present was measured using the Qubit fluorometer.

In vivo DNA samples from original and transplanted ERS tumors were also analyzed using a DNA methylation array. For this, only DNA quantification, following the previously described protocol, was performed, as the DNA had already been extracted from mice ERS tumors.

6. Inoculation of HCC827 Cells

To obtain DNA samples from ESS tumors from mice, 5×10^6 HCC827 cells were injected into the dorsal flank of six 8-12 weeks old C57BL/6 *Rag2*^{-/-}; *IL2rg*^{-/-} immunodeficient mice.

HCC827 cells were incubated with trypsin-EDTA 1x solution (Alfagene Cat# 15400054) for the appropriate amount of time and the resulting cell suspension was collected to a 50 mL falcon and centrifuged at 1200 rpm for 5 minutes. Then, the supernatant was discarded, and the pellet resuspended in RPMI-1640. Live cells were counted using trypan blue dye and cell counting slides in a TC20™ automated cell counter (Bio-Rad). The appropriate volume containing the desired cell concentration was once again centrifuged, the supernatant was discarded, and the pellet resuspended in NaCl (B. Braun Cat# 3570130) and transferred to an eppendorf.

The mice inoculated with HCC827 cells were monitored and the tumor size measured with a caliper three times a week. Tumor volume was calculated according to the formula $(\text{length} \times \text{width}^2)/2$. Tumors were excised when they reached a volume of $\approx 1000 \text{ mm}^3$ and fixed in 10% formalin for subsequent DNA extraction. Animals were euthanized at this point or when a humane endpoint was reached.

7. DNA Methylation Analysis

The DNA samples collected were shipped to Hospital Clínico Universitario de Santiago (Santiago de Compostela, Spain) and analyzed using the DNA methylation array Infinium™ Methylation EPIC v2.0 BeadChip. This determines the methylation status of the sample DNA at specific CpG sites targeted by the array. First, the DNA samples were bisulfite-converted using the EZ-96 DNA Methylation Kit (Zymo Research) following the manufacturer's instructions. The bisulfite-converted DNA from mice tumor samples was also

treated with the Infinium HD FFPE Restore Kit (Illumina) according to the manufacturer's instructions. This allows the subsequent hybridization of the sample DNA to the BeadChips of the Infinium™ Methylation EPIC v2.0 BeadChip (Illumina), following the manufacturer's protocol. After hybridization, the BeadChips were analyzed using an iScan system (Illumina) in order to assess the methylation status of cytosines of the sample DNA at each specific site.

8. Concentration-Response Experiments

HEK293T cells (ATCC Cat# CRL-3216™) were used as a control to verify the effectiveness of the lentiviral particles used for the transduction of HCC827 cells. Next, the puromycin (Sigma-Aldrich Cat# P8833-10MG) and polybrene concentrations and the multiplicity of infection (MOI) were determined for HCC827 cells for the transduction protocol.

To establish the optimal puromycin concentration, 1.6×10^4 HCC827 cells were plated in a 96-well plate and exposed to puromycin concentrations ranging from 0.5 to 10 µg/mL every 2-3 days until complete or near-complete cell death occurred. The minimum concentration of puromycin that visually caused complete cell death after 4-7 days was selected.

To settle the polybrene concentration, 0.5×10^5 HCC827 cells were seeded in a 24-well plate and exposed to polybrene concentrations ranging from 2 to 10 µg/mL. After 24 hours, cell viability was assessed, and the highest concentration of polybrene that visually did not compromise cell viability was selected.

Finally, to determine the MOI for HCC827 cells, various MOI values (1 to 12), corresponding to different volumes of lentiviral particles, were used to supplement HCC827 cells plated at a concentration of 0.5×10^5 in a 24-well plate. After 48-72 hours, the MOI resulting in the highest number of tGFP fluorescent cells, which indicates successful incorporation of the viral vector, that did not compromise cell viability was chosen.

9. Transduction of HCC827 Cells

HCC827 cells were transduced using lentiviral vectors (OriGene Cat# TL314183V) containing three main functional elements: a short hairpin RNA (shRNA) expression cassette, driven by an U6 promoter, to reduce *CAV1* gene expression in transduced cells; a puromycin resistance marker, driven by a SV40 promoter, to positively select antibiotic-

resistant cells; and a turbo green fluorescent protein (tGFP) reporter gene, driven by a CMV promoter, to serve as a readout for successful transduction. The kit with the lentiviral particles contains five vials: one with the negative control, which are lentiviral particles carrying a scrambled sequence cassette without a target gene, that will activate the RNA interference (RNAi) pathway, and carrying the tGFP reporter gene and the puromycin selection marker (*Application Guide: HUSH shRNA Plasmids and Lenti-Particles*); and four lentiviral vectors targeting multiple splice variants at the *CAV1* gene locus (*Caveolin 1 (CAV1) Human shRNA Lentiviral Particle (Locus ID 857)*, 2023).

0.5×10^5 HCC827 cells were seeded in a 24-well plate until 50% confluency. Upon reaching 50% confluency, the culture media was removed from the wells and replaced with 500 μ L of a mix of new media, lentiviral particles (at a MOI of 12) and polybrene (at a final concentration of 6 μ g/mL). Polybrene neutralizes the cell surface charge and allows the binding of the viral particles to the cell membrane, thus enhancing transduction efficiency (*Application Guide: HUSH shRNA Plasmids and Lenti-Particles*). The 24-well plate was then incubated in a 37°C, 5% CO₂ incubator for 18-20 hours and, after this period, the medium containing lentiviral particles was replaced with fresh complete culture medium. The cells grew until they reached 70-80% confluency and then puromycin (at a final concentration of 0.5 μ g/mL) was added to the media. The puromycin-containing media was changed every 2-3 days until resistant colonies started to appear. Resistant colonies were selected and seeded in a 6-well plate.

10. Flow Cytometry of Transduced HCC827 Cells

The cellular conditions submitted to flow cytometry were the following: CAV1-unstained HCC827 cells, HCC827 parental cells, HCC827 cells transduced with the scramble, HCC827 cells transduced with construct #1 and HCC827 cells transduced with construct #2. These cells were incubated with trypsin-EDTA 1x solution (Alfagene Cat# 15400054) for the appropriate amount of time and the resulting cell suspension was collected to 15 mL falcons and the cells counted using a TC20™ automated cell counter (Bio-Rad). $0.5-1 \times 10^6$ cells of each condition were collected into new 15 mL falcons and centrifuged at 1200 rpm for 5 minutes. From now on, the protocol was carried out at 4°C (on ice). The supernatant was discarded and the cells resuspended in FACS buffer (PBS 1x + 2% FBS) and transferred to a 96-well plate. Another centrifugation (at 4°C), at 1500 rpm for 3 minutes, was performed, the supernatant discarded and fixation buffer (Invitrogen Cat# 00-5123-43), diluted in fixation diluent (Invitrogen Cat# 00-5223-56), added to each well. The 96-well plate was incubated in the dark for 30 minutes. Following this period, a

permeabilization buffer (Invitrogen Cat# 00-8333-56), diluted in type II H₂O, was added to the wells and mixed with the other solutions by up-and-down motions with the automatic pipette. The plate was centrifuged (at 4°C) at 1500 rpm for 3 minutes and, afterwards, the supernatant was discarded. Then, the anti-CAV1 antibody conjugated with the phycoerythrin (PE) fluorophore (Abcam Cat# ab212007, EPR15554 clone), diluted 1:300 in FACS buffer in accordance with the number of cells measured before ($\approx 50 \mu\text{L}$ of FACS buffer for $0.5\text{-}1 \times 10^6$ cells), was added to the wells and the plate was incubated in the dark for another 30 minutes (the CAV1-unstained HCC827 cells were incubated with FACS buffer only). Next, FACS buffer was added to each well and the plate was centrifuged (at 4°C) at 1500 rpm for 3 minutes. The supernatant was discarded and the cells resuspended in FACS buffer and transferred to polystyrene tubes with a cell strainer cap in order to be analyzed using the BD FACS Canto™ II flow cytometer. The results obtained were processed using the FlowJo software.

11. Statistical Analysis

For the IC₅₀ and MTT assays, the statistical analysis was performed using GraphPad Prism 9 (GraphPad Prism, RRID:SCR_002798). The data is shown as means $\pm$ standard deviation (SD). For the IC₅₀ assays, the statistical analysis conducted was a nonlinear regression (using the least squares method) of a normalized dose-response curve in order to estimate the IC₅₀ values of the drugs for the cell lines in study. For the MTT assays, outliers were identified and excluded, and all absorbance values were adjusted using the mean value of the blank samples. The data was analyzed using one-way and two-way ANOVA (analysis of variance) tests and subsequent multiple comparison post-hoc tests (e.g., Bonferroni test) to determine statistically significant differences ($p\text{-value} < 0.05$).

The results obtained with the DNA methylation array were processed within the R statistical environment using packages available in Bioconductor. The minfi package was used to perform quality control and data preprocessing. The methylation score of each CpG was expressed as β -values, which were normalized for color bias and background level adjustments. Additionally, quantile normalization was applied across BeadChips. Samples with a mean detection $p\text{-value} < 0.01$ were considered valid for analysis. The resulting β -values, ranging from 0 (no methylation) to 1 (complete methylation), underwent a two-step filtering process. This process involved excluding SNPs and unreliable β -values with a detection $p\text{-value} > 0.01$. After this filtering step, the remaining CpGs were considered valid for the study. Differential methylation analysis between groups was performed using the

limma package. P-values were adjusted for multiple testing using the Benjamini-Hochberg method in order to control the false discovery rate (FDR).

Results

1. Cell Viability Assays

As mentioned earlier, previous results from our group revealed that PI3K presented an increased expression and that AKT1 and ERK1/2 presented an increased activation in ERS tumors from mice, compared to ESS tumors. As such, HCC827 cells before and after acquiring the resistance phenotype were treated with the PI3K inhibitor wortmannin, with the AKT inhibitor miransertib and with the ERK inhibitor SCH772984 in order to select a concentration of these drugs to combine with erlotinib treatment and check if these dual inhibitions resensitize HCC827 cells displaying the resistance phenotype to the EGFR-TKI therapy. For this, MTT assays were performed. A MTT assay is a colorimetric assay widely used to assess the cytotoxicity of drugs since it allows the measurement of metabolically active cells through the reduction of the yellow MTT to purple formazan crystals.

1.1. PI3K

The comparison between mice's ESS and ERS tumors using RNA-seq showed a statistically significant 1.92-fold increase of *PIK3CB*, the p110 β catalytic subunit of class IA of PI3Ks, in ERS tumors exhibiting the resistance phenotype to erlotinib treatment. Additionally, *CAV1*, a protein previously identified as potentially playing a critical role in the development of resistance to therapy, has been shown to participate in the activation of the PI3K-AKT signaling pathway (Wang et al., 2018; Yang et al., 2016). Given the upregulation and key role of PI3K in the signaling pathway triggered through *CAV1* overexpression, the drug wortmannin was chosen to target PI3K to check if this inhibition rescues sensitivity to erlotinib treatment in HCC827 cells that display the resistance phenotype. Wortmannin is a non-isoform specific irreversible inhibitor of PI3K that forms a covalent adduct in the lysine residue 802 in the ATP binding pocket of PI3K (Elmenier et al., 2019; George et al., 2019), thus not allowing the activation of this kinase.

First, the HCC827 parental cell line, used as a control for the experiment, and HCC827 cells that display the resistance phenotype were incubated with different concentrations of wortmannin during 24 and 48 hours to perform IC₅₀ assays to choose a concentration of wortmannin to be used. For this, both cell lines were treated with increasing concentrations of wortmannin ranging from 1 μ M to 120 μ M based on literature (Ihara et al., 2020).

Given that the drug's effect was less evident in the 24-hour assay, the 48-hour IC₅₀ assays were considered.

For both cell lines, the relative cell survival exhibited a dose-dependent effect, displaying an exponential decrease with increasing concentrations of wortmannin. By directly assessing the cell viability corresponding to different concentrations of wortmannin, it was possible to select the concentration of 5 μM of wortmannin to combine with erlotinib therapy for HCC827 cells that display the resistance phenotype. At the maximal dose of 120 μM of wortmannin, cell viability reached 25.1% for HCC827 cells and 18.1% for HCC827 cells that display the resistance phenotype, highlighting a cytotoxic effect of this drug (Figure 9).

Through nonlinear regression (using the least squares method) of a normalized dose-response curve, the IC₅₀ values of wortmannin for the two cell lines in study were estimated. For HCC827 cells, the coefficient of determination (R^2) measured is 0.9643, which means that this model is good at fitting the data and that 96.43% of the variation in the relative cell survival is explained by the concentration of wortmannin. Moreover, the model indicated an IC₅₀ of 15.09 μM for these cells. For HCC827 cells that display the resistance phenotype, the R^2 measured is 0.9632, which also means that this model is a good fit for the data and that 96.32% of the variation in the relative cell survival is explained by the concentration of wortmannin. In addition, this model indicated an IC₅₀ of 10.30 μM for this cell line.

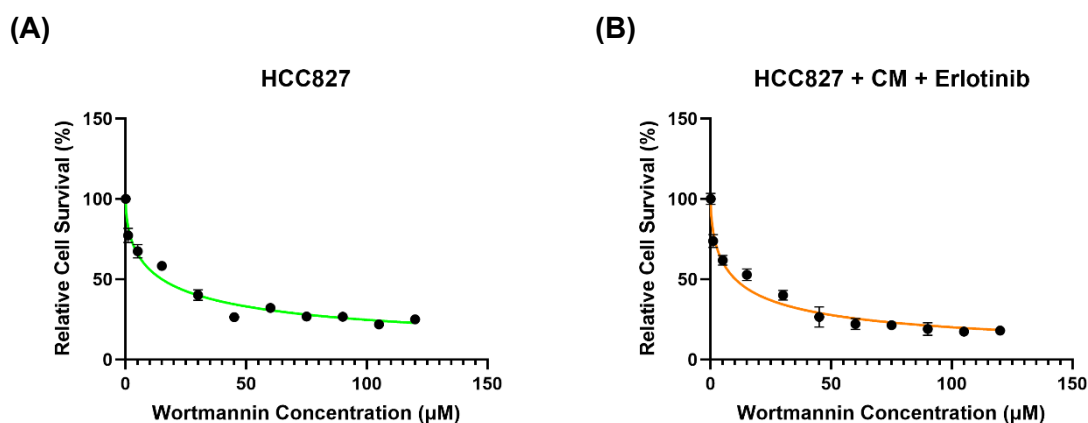


Figure 9 – Wortmannin IC₅₀ assays for the HCC827 erlotinib-sensitive cells and HCC827 cells presenting the resistance phenotype. The HCC827 erlotinib-sensitive cells (A) and the HCC827 cells presenting the resistance phenotype (B) exhibit an exponential decrease on cell viability when treated with increasing concentrations of the PI3K inhibitor wortmannin. Each value is presented as the mean $\pm$ SD (n = 6, six replicates for each wortmannin concentration were used in the 48-hour IC₅₀ assay).

1.1.1. Wortmannin rescues sensitivity to erlotinib treatment in HCC827 cells that display the resistance phenotype

MTT assays were performed, using the HCC827 parental cell line as a control and using HCC827 cells that display the resistance phenotype, to check if the combination treatment of erlotinib and 5 μ M of wortmannin rescued sensitivity to the EGFR-TKI therapy in cells presenting the resistance phenotype. The increased sensitivity to erlotinib with the addition of the PI3K inhibitor would point to the resistance phenotype possibly occurring due to the activation of signaling pathways involving PI3K downstream of caveolin.

The HCC827 parental cell line and the HCC827 cell line that exhibits the resistance phenotype were plated at a concentration of 1×10^4 cells per well for 24 hours. Afterwards, the cells were incubated in RPMI containing DMSO or wortmannin for an additional 24 hours. On the following day, the cells were once again supplemented with either wortmannin or DMSO. In addition, erlotinib at a concentration of 0.1 or 1 μ M was added to all cells, which were then incubated for 48 hours, considering previous IC₅₀ assays. Moreover, the erlotinib solvent, methylcellulose, and the drug's solvent, DMSO, showed no statistically significant cytotoxic effect in cell viability (therefore are not represented on the graphs below).

Regarding HCC827 cells, we can see that, when comparing the control condition with the conditions treated with 0.1 μ M or 1 μ M of erlotinib, there is a statistically significant decrease in relative cell survival from 92% to 39% and 44%, respectively, as expected considering that these cells are sensitive to erlotinib therapy (**Figure 10A**). Additionally, this is also observed when comparing the wortmannin condition with the conditions treated with the combination of 0.1 μ M or 1 μ M of erlotinib and 5 μ M of wortmannin, with the relative cell survival falling from 96% to 43% and 41%, respectively (**Figure 10A**). When simultaneously comparing the effect of wortmannin in HCC827 cells supplemented with erlotinib (**Figure 10B**), there is not a statistically significant difference on cell viability and so, overall, it was shown that the cell death occurring in HCC827 cells is caused mainly by erlotinib treatment.

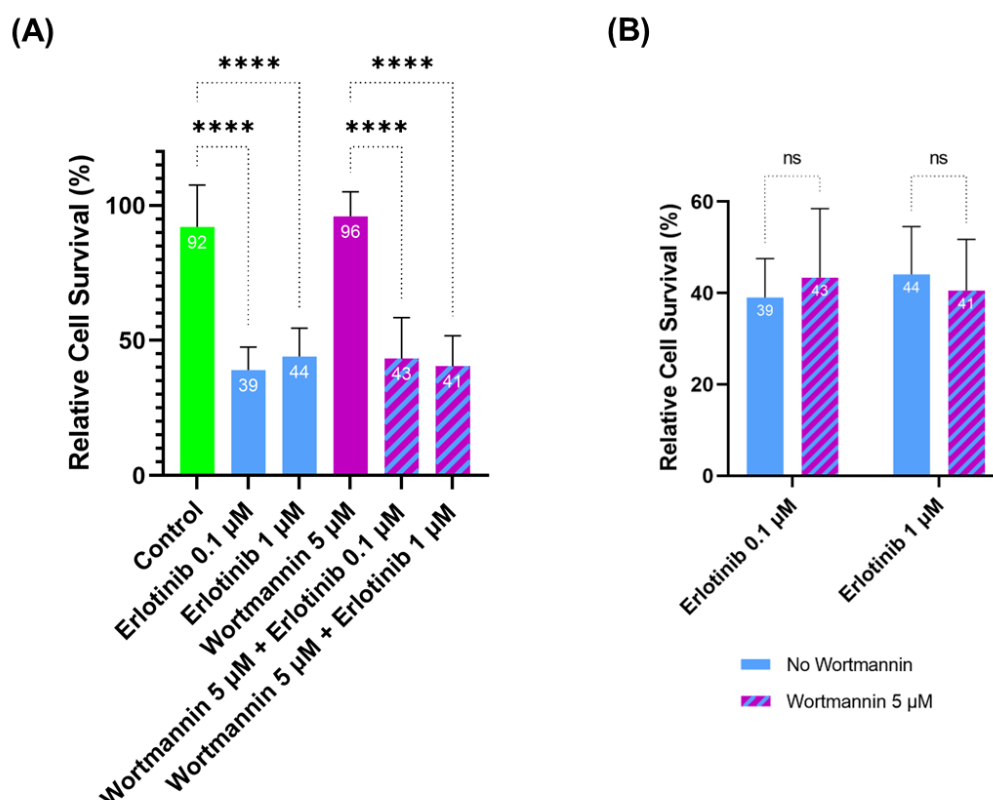


Figure 10 – The influence of the PI3K inhibitor wortmannin on the viability of HCC827 erlotinib-sensitive cells. (A) There is a significant (adjusted p-value < 0.0001) decrease on cell viability from the control HCC827 cells to HCC827 cells treated with 0.1 μ M or 1 μ M of erlotinib. There is also a significant (adjusted p-value < 0.0001) decrease on cell viability from the wortmannin condition to HCC827 cells treated with 5 μ M of wortmannin and 0.1 μ M or 1 μ M of erlotinib. **(B)** There is not a significant change on cell viability in HCC827 cells treated with erlotinib in comparison to cells treated with erlotinib and wortmannin. Each value is presented as the mean $\pm$ SD (n = 12, twelve replicates of each condition were used for all assays). Statistical significance was tested using one-way and two-way ANOVA: ns = non-significant (p-value is equal to or greater than 0.05); **** = p-value is less than 0.0001.

On the other hand, when comparing the control condition with the conditions treated with 0.1 μ M and 1 μ M of erlotinib, we can see that HCC827 cells displaying the resistance phenotype do not present a statistically significant difference in relative cell survival when treated with 1 μ M of erlotinib, as expected considering that these cells are resistant to erlotinib therapy, although this is not observed with 0.1 μ M of erlotinib (**Figure 11A**). Moreover, when these cells are treated with 5 μ M of wortmannin the relative cell viability falls to 41%. However, the combination of this drug with 0.1 μ M or 1 μ M of erlotinib, decreases the relative cell survival significantly more: the relative cell survival falls from 41% to 30%, with an adjusted p-value of less than 0.0001, for 0.1 μ M erlotinib, and from 41% to 37%, with an adjusted p-value of 0.0013, for 1 μ M erlotinib in relation to the wortmannin condition (**Figure 11A**). When simultaneously comparing the effect of wortmannin in HCC827 cells displaying the resistance phenotype supplemented with erlotinib (**Figure 11B**), there is a statistically significant decrease on cell viability when these cells are treated with the combination of 5 μ M of wortmannin and 0.1 μ M of erlotinib (adjusted p-value <

0.0001) or with 5 μM of wortmannin and 1 μM of erlotinib (adjusted p-value < 0.0001), in comparison to the treatment with erlotinib in monotherapy. Overall, these results indicate that the combination treatment of erlotinib and wortmannin might resensitize HCC827 cells that display the resistance phenotype for the EGFR-TKI therapy.

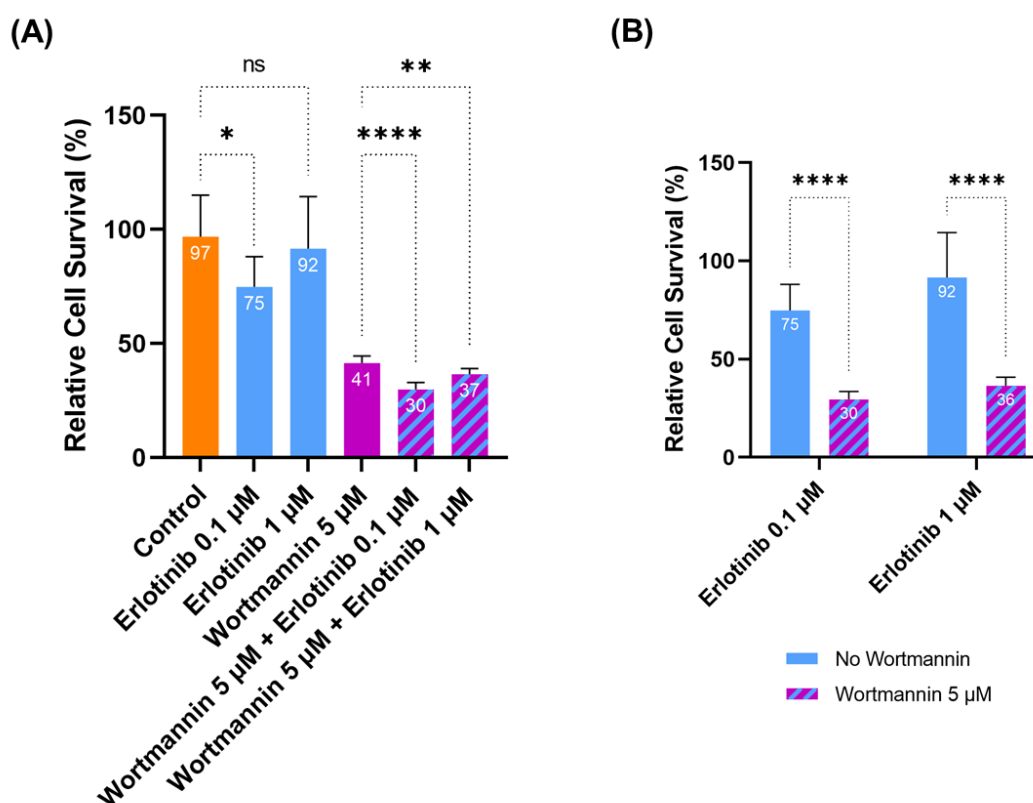


Figure 11 – The combination treatment of erlotinib and wortmannin might rescue sensitivity to erlotinib. (A) There is no significant difference on cell viability between the control HCC827 cells that display the resistance phenotype and the condition treated with 1 μM of erlotinib. There is a significant decrease on cell viability from the wortmannin condition to HCC827 cells displaying the resistance phenotype treated with 5 μM of wortmannin and 0.1 μM (adjusted p-value < 0.0001) or 1 μM (adjusted p-value = 0.0013) of erlotinib. (B) There is a significant decrease on cell viability in cells treated with 0.1 μM of erlotinib or 1 μM of erlotinib (both with an adjusted p-value < 0.0001) and wortmannin in comparison to cells treated with erlotinib in monotherapy. Each value is presented as the mean $\pm$ SD (n = 12, twelve replicates of each condition were used for all assays). Statistical significance was tested using one-way and two-way ANOVA: ns = non-significant (p-value is equal to or greater than 0.05); * = p-value is less than 0.05 and greater than 0.03; ** = p-value is equal to or less than 0.03 and equal to or greater than 0.001; **** = p-value is less than 0.0001.

1.2. AKT

When comparing mice's ESS and ERS tumors through a phospho-kinase array, AKT1 showed significantly (p-value = 0.043490) different phosphorylation levels in threonine 308 (T308) in the kinase domain of AKT, indicating its increased activation in ERS tumors exhibiting the resistance phenotype, as previously denoted. Therefore, the drug miransertib, a selective and allosteric pan-AKT inhibitor (Afrin et al., 2018), was chosen to

target AKT in order to investigate if this inhibition rescues sensitivity to erlotinib treatment in HCC827 cells that display the resistance phenotype. Miransertib acts in two ways to compromise AKT activity: it targets the unphosphorylated/inactive form of AKT and prevents its translocation to the plasma membrane; and it promotes the dephosphorylation of active AKT located in the plasma membrane (Yu et al., 2015).

First, the HCC827 parental cell line, used as a control for the experiment, and HCC827 cells that display the resistance phenotype were incubated with different concentrations of miransertib during 24 and 48 hours to perform IC₅₀ assays to choose a concentration of miransertib to be used. For this, both cell lines were treated with increasing concentrations of this drug ranging from 1 μ M to 60 μ M based on literature (Afrin et al., 2018; Chen et al., 2022; Wu et al., 2023).

Given that the drug's effect was less evident in the 24-hour assay, the 48-hour IC₅₀ assays were considered.

For HCC827 cells treated with increasing concentrations of miransertib, the relative cell survival fluctuated around 100% without a clear tendency (**Figure 12A**). Consequently, the nonlinear regression curve did not fit the data well ($R^2 = -0.08228$), thus not being possible to calculate the IC₅₀ value for this drug. At the maximal dose tested (60 μ M of miransertib), 74.8% of HCC827 cells were alive.

For HCC827 cells that present the resistance phenotype, the nonlinear regression curve fit the data slightly better ($R^2 = 0.6738$). However, the relative cell survival trend does not match with the increasing concentrations of miransertib (**Figure 12B**) since there is considerable cell death at lower concentrations of miransertib, which decreases with increasing concentrations of miransertib until a point where it starts increasing exponentially. Moreover, the model indicated an IC₅₀ of 43.50 μ M for these cells and, at the maximal dose tested (60 μ M of miransertib), 13.8% of HCC827 cells that display the resistance phenotype were alive. By directly assessing the cell viability corresponding to different concentrations of miransertib, it was possible to select the concentration of 13 μ M of miransertib to combine with erlotinib therapy for HCC827 cells that display the resistance phenotype.

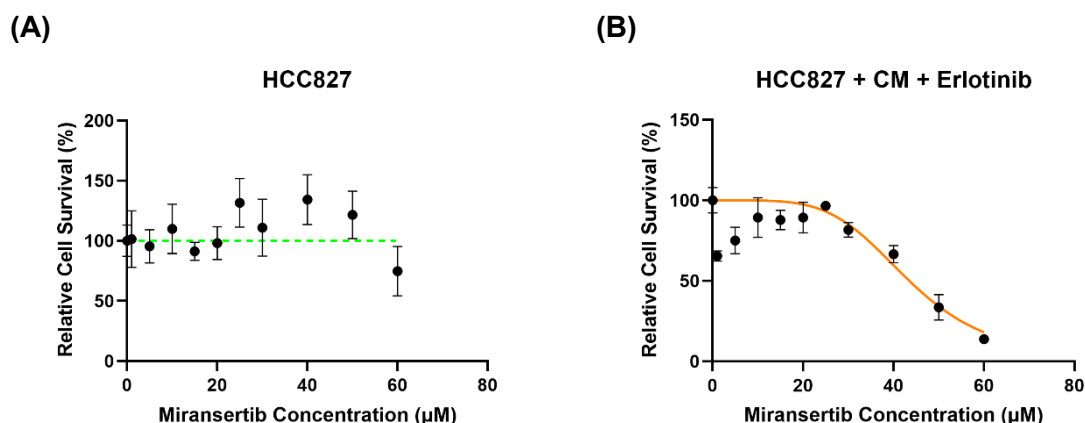


Figure 12 – Miransertib IC₅₀ assays for HCC827 erlotinib-sensitive cells and HCC827 cells presenting the resistance phenotype. (A) Miransertib appears to not affect HCC827 cells' survival since the relative cell survival for the control HCC827 cells fluctuated around 100% without a clear tendency when these cells were treated with increasing concentrations of the drug. **(B)** HCC827 cells that exhibit the resistance phenotype displayed a response to increasing concentrations of miransertib. Each value is presented as the mean $\pm$ SD ($n = 6$, six replicates for each miransertib concentration were used in the 48-hour IC₅₀ assay).

1.2.1. Miransertib does not resensitize HCC827 cells that display the resistance phenotype for erlotinib treatment

MTT assays were performed, using the HCC827 parental cell line as a control and using HCC827 cells that display the resistance phenotype, to check if the combination treatment of erlotinib and 13 μ M of miransertib rescued sensitivity to the EGFR-TKI therapy in cells presenting the resistance phenotype possibly due to the activation of signaling pathways involving AKT1 downstream of caveolin.

In a procedure similar to what was described previously when assessing wortmannin's effect, the cells were incubated with RPMI containing DMSO or miransertib and, afterwards, exposed to the drug/DMSO and erlotinib for 48 hours, considering previous IC₅₀ assays. Moreover, the erlotinib solvent, methylcellulose, and the drug's solvent, DMSO, showed no statistically significant cytotoxic effect in cell viability (therefore are not represented on the graphs below).

For HCC827 cells, we can see that, when comparing the control condition with the conditions treated with 0.1 μ M and 1 μ M of erlotinib, there is a statistically significant decrease in relative cell survival from 117% to 39% and 44%, respectively, as expected considering that these cells are sensitive to erlotinib therapy (**Figure 13A**). This is also observed when comparing the miransertib condition with the conditions treated with the combination of 13 μ M of miransertib and 0.1 μ M or 1 μ M of erlotinib, in which the relative cell survival exhibits a statistically significant decrease from 185% to 55% and 77%, respectively (**Figure 13A**). When simultaneously comparing the effect of miransertib in HCC827 cells supplemented with erlotinib (**Figure 13B**), there is a significant difference in

the relative cell survival between HCC827 cells treated with 1 μM of erlotinib in monotherapy to its combination with 13 μM of miransertib, however, it is possible to say that, overall, the cell death occurring in HCC827 cells is being mainly caused by the erlotinib treatment.

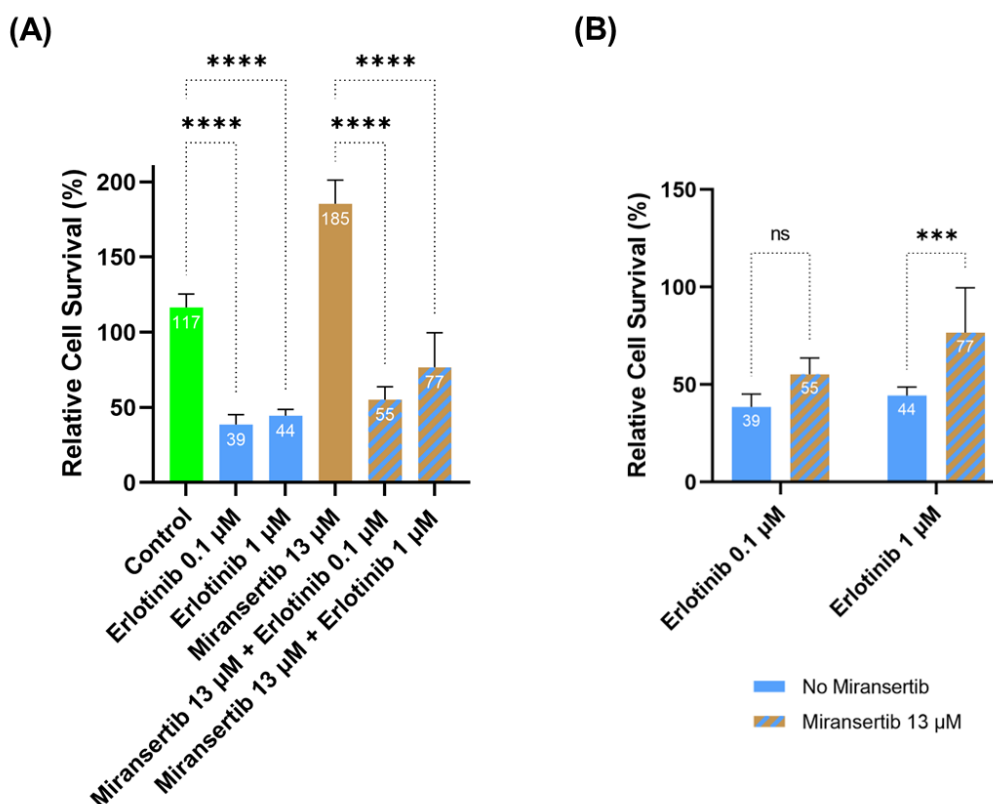


Figure 13 – Difference in HCC827 cells survival when treated with erlotinib and miransertib. (A) The treatment of HCC827 cells with 0.1 μM and 1 μM erlotinib show a significant decrease in relative cell survival compared to the control condition. Plus, the treatment of HCC827 cells with erlotinib and 13 μM of miransertib significantly reduces HCC827 cell viability when compared to the miransertib condition. (B) Treatment of HCC827 cells with 1 μM of erlotinib and miransertib showed significantly (adjusted p-value = 0.0006) more viability when compared to 1 μM erlotinib in monotherapy. Each value is presented as the mean $\pm$ SD (n = 6, six replicates of each condition were used for all assays). Statistical significance was tested using one-way and two-way ANOVA: ns = non-significant (p-value is equal to or greater than 0.05); *** = p-value is less than 0.001; **** = p-value is less than 0.0001.

Regarding the HCC827 cells that display the resistance phenotype, we can see that, when comparing the control and miransertib conditions with the conditions where the cells were treated with erlotinib in monotherapy and erlotinib combined with miransertib (**Figure 14A**) respectively, there is not a statistically significant difference in relative cell survival. This observation, when cells were treated with erlotinib, is consistent with the fact that these cells are erlotinib-resistant, thus erlotinib therapy does not alter relative cell survival here. Regarding the cells treated with a combination of miransertib and erlotinib, the absence of statistical significance between the miransertib condition and the combination treatment conditions might indicate that treating these cells with miransertib does not render them sensitive to erlotinib. This is also evidenced in **Figure 14B**, where there is not a statistically

significant difference on cell viability when HCC827 cells that exhibit the resistance phenotype are treated with erlotinib and miransertib compared to erlotinib alone.

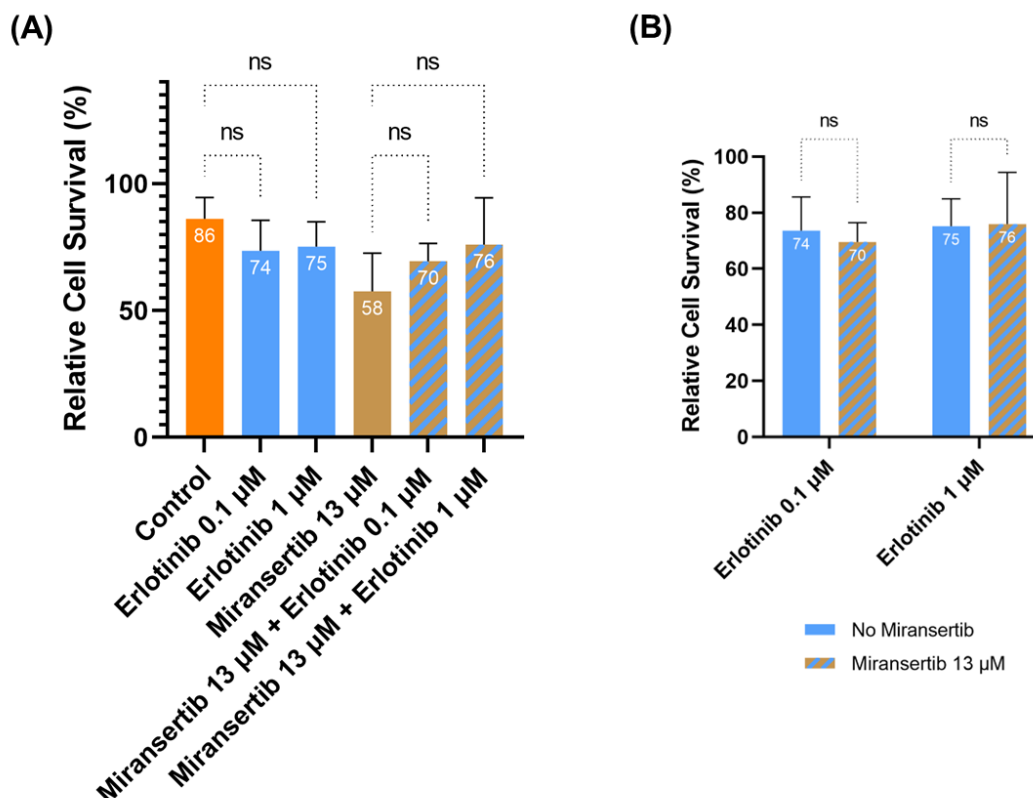


Figure 14 – The combination treatment of erlotinib and miransertib does not influence the survival of HCC827 cells that display the resistance phenotype. (A) Treatment of HCC827 cells presenting the resistance phenotype with increasing concentrations of erlotinib does not lead to a statistically significant difference in cell survival when compared to the control condition. Plus, the treatment of HCC827 cells presenting the resistance phenotype with erlotinib and miransertib showed no statistically significant differences in cell survival when compared to the miransertib condition. (B) Viability of HCC827 cells presenting the resistance phenotype showed no statistically significant differences when treated with 0.1 μ M or 1 μ M erlotinib and miransertib. Each value is presented as the mean $\pm$ SD (n = 6, six replicates of each condition were used for all assays). Statistical significance was tested using one-way and two-way ANOVA: ns = non-significant (p-value is equal to or greater than 0.05).

1.3. ERK

As previously mentioned, a phospho-kinase array performed in ESS and ERS tumors from mice revealed that ERK1 and ERK2 showed significantly (p-value = 0.032739) different phosphorylation levels in its activation loop, specifically in threonine 202 and tyrosine 204 (T202/Y204) and in threonine 185 and tyrosine 187 (T185/Y187), respectively. This shows ERK1/2 increased activation in ERS tumors, which exhibit the resistance phenotype. As such, the chemical inhibitor SCH772984, a selective inhibitor of both ERK1 and ERK2 (Wong et al., 2014), was chosen to investigate if this inhibition, along with erlotinib treatment, rescues sensitivity to erlotinib in HCC827 cells that display the

resistance phenotype. SCH772984 inhibits ERK1/2 activity by preventing the phosphorylation of T202/Y204 of ERK1 and of T185/Y187 of ERK2 and consequent activation of these kinases by MEK1/2 (Morris et al., 2013).

First, the HCC827 parental cell line, used as a control for the experiment, and HCC827 cells that display the resistance phenotype were incubated with different concentrations of SCH772984 during 24 and 48 hours to perform IC₅₀ assays to choose a concentration of SCH772984 to be used. For this, both cell lines were treated with increasing concentrations of this drug ranging from 1 μ M to 30 μ M based on literature (Hayes et al., 2016).

Given that the drug's effect was less evident in the 24-hour assay, the 48-hour IC₅₀ assays were considered.

As happened with the AKT inhibitor miransertib, the relative survival of HCC827 cells treated with increasing concentrations of SCH772984 fluctuated around 100% without a trend (**Figure 15A**). As such, the nonlinear regression curve determined did not fit the data well ($R^2 = 0.007713$) and so it was not possible to assess the IC₅₀ value of SCH772984 for this cell line. Moreover, only 18.6% of HCC827 cells died when treated with 30 μ M of SCH772984, the maximal dose tested.

For HCC827 cells that display the resistance phenotype, there is an exponential decrease on cell viability with the increase of SCH772984 concentration (**Figure 15B**). The nonlinear regression curve fit the data well ($R^2 = 0.9061$), which means that 90.61% of the variation in the relative cell survival is explained by the concentration of SCH772984. The IC₅₀ value was interpolated and corresponded to 9.181 μ M of SCH772984. Additionally, by directly assessing the cell viability corresponding to different concentrations of SCH772984, it was possible to select the concentration of 5 μ M of SCH772984 to combine with erlotinib therapy for HCC827 cells that display the resistance phenotype. At the maximal dose of 30 μ M of SCH772984, cell viability reached 26.6% for this cell line.

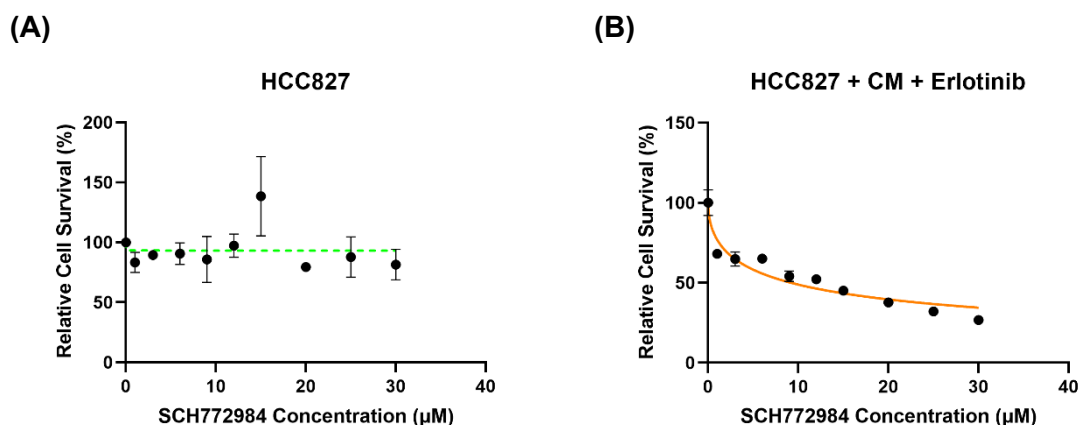


Figure 15 – SCH772984 IC₅₀ assay for HCC827 erlotinib-sensitive cells and HCC827 cells presenting the resistance phenotype. (A) SCH772984 appears to not affect HCC827 cells' survival since the relative cell survival for the control HCC827 cells was close to 100% with the treatment with increasing concentrations of the drug. (B) The relative survival of HCC827 cells presenting the resistance phenotype show a decrease when treated with increasing concentrations of SCH772984. The concentration of 5 µM of SCH772984 was selected to be used in combination with erlotinib. Each value is presented as the mean $\pm$ SD (n = 6, six replicates for each SCH772984 concentration were used for the 48-hour IC₅₀ assay).

1.3.1. HCC827 cells that display the resistance phenotype can be resensitized to erlotinib treatment when treated in combination with the ERK inhibitor SCH772984

MTT assays were performed, using the HCC827 parental cell line as a control and using HCC827 cells that display the resistance phenotype, to check if the combination treatment of erlotinib and 5 µM of SCH772984 rescued sensitivity to the EGFR-TKI therapy in cells presenting the resistance phenotype highlighting the possibility that resistance can occur due to the activation of signaling pathways involving ERK1/2 downstream of caveolin.

DMSO or SCH772984 were used to supplement growing HCC827 parental cells and HCC827 cells that display the resistance phenotype once they were plated as described before. Afterwards, the cells were incubated with the drug/DMSO and erlotinib for 48 hours, considering previous IC₅₀ assays. Moreover, the erlotinib solvent, methylcellulose, and the drug's solvent, DMSO, showed no statistically significant cytotoxic effect in cell viability (therefore are not represented on the graphs below).

For HCC827 cells, we can see that there is a statistically significant decrease in relative cell survival when these cells are treated with erlotinib in comparison to the control condition (**Figure 16A**), which was expected, considering that this cell line is sensitive to erlotinib therapy. Furthermore, when the viability of cells growing in the presence of SCH772984 is compared to the viability of HCC827 cells treated with 5 µM of SCH772984 and 0.1 µM or 1 µM of erlotinib, a significant decrease in cell survival is observed (**Figure 16A**). When simultaneously comparing the effect of SCH772984 in HCC827 cells supplemented with erlotinib (**Figure 16B**), we can see that there is a statistically significant

difference in cell viability, in which the cells treated with the combination treatment of erlotinib and 5 μ M of SCH772984 show less survival.

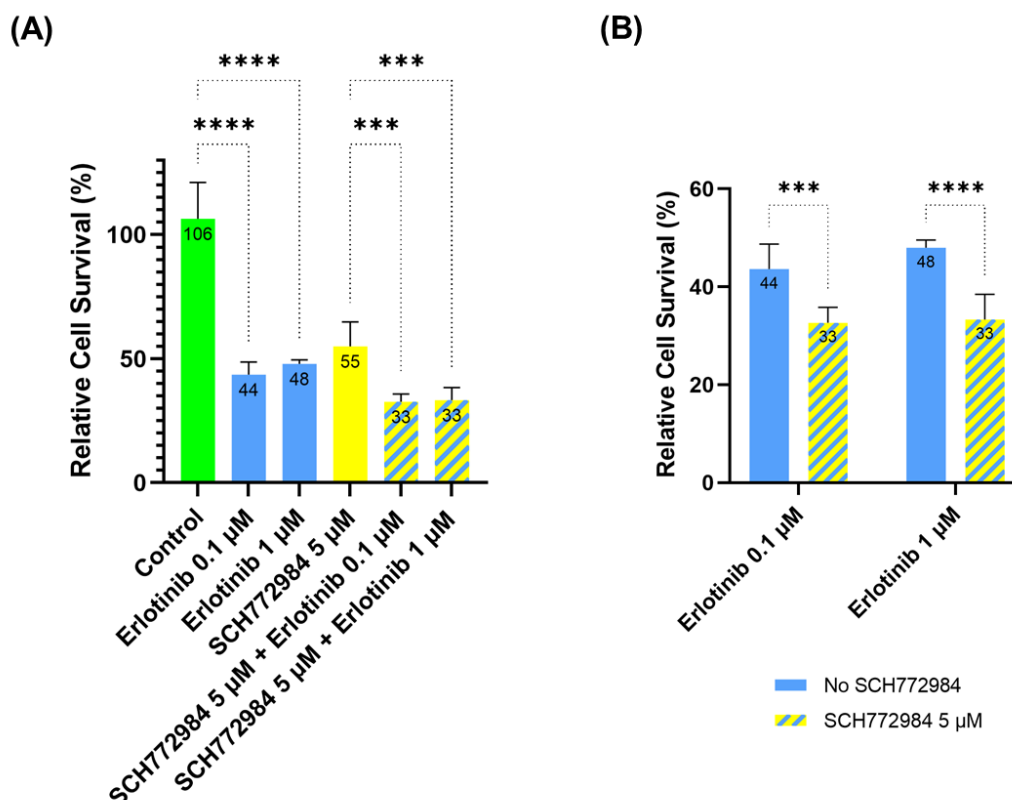


Figure 16 – HCC827 cells remain sensitive to erlotinib when supplemented with the ERK inhibitor SCH772984. (A) Erlotinib-sensitive HCC827 cells treated with 0.1 μ M and 1 μ M erlotinib show a decrease in relative cell survival compared to the control condition. Plus, the combination of erlotinib and 5 μ M of SCH772984 significantly reduces the relative survival of HCC827 cells when compared to the SCH772984 condition. (B) There is a significant decrease on HCC827 cells viability when they are treated with 0.1 μ M of erlotinib (adjusted p-value = 0.0004) or 1 μ M of erlotinib (adjusted p-value < 0.0001) and SCH772984 in comparison to the treatment with erlotinib in monotherapy. Each value is presented as the mean $\pm$ SD (n = 6, six replicates of each condition were used for all assays). Statistical significance was tested using one-way and two-way ANOVA: *** = p-value is less than 0.001. **** = p-value is less than 0.0001.

Regarding HCC827 cells displaying the resistance phenotype, there is not a statistically significant change in the relative survival when comparing the control condition to the treatment with erlotinib (**Figure 17A**), which reflects the fact that these cells are resistant to this EGFR-TKI therapy. However, when HCC827 cells displaying the resistance phenotype are treated with both 5 μ M of SCH772984 and 0.1 μ M or 1 μ M of erlotinib, cell viability significantly decreases from 41% (SCH772984 only condition) to 34% and 30%, respectively (**Figure 17A**). Moreover, looking at the results put together side by side (**Figure 17B**), the relative survival of HCC827 cells displaying the resistance phenotype is significantly lower with the combination treatment: 34% when treated with 0.1 μ M of erlotinib and 5 μ M of SCH772984 and 30% when treated with 1 μ M of erlotinib and 5 μ M of SCH772984. These findings suggest that the combination treatment of erlotinib and

SCH772984 might resensitize HCC827 cells exhibiting the resistance phenotype to erlotinib therapy.

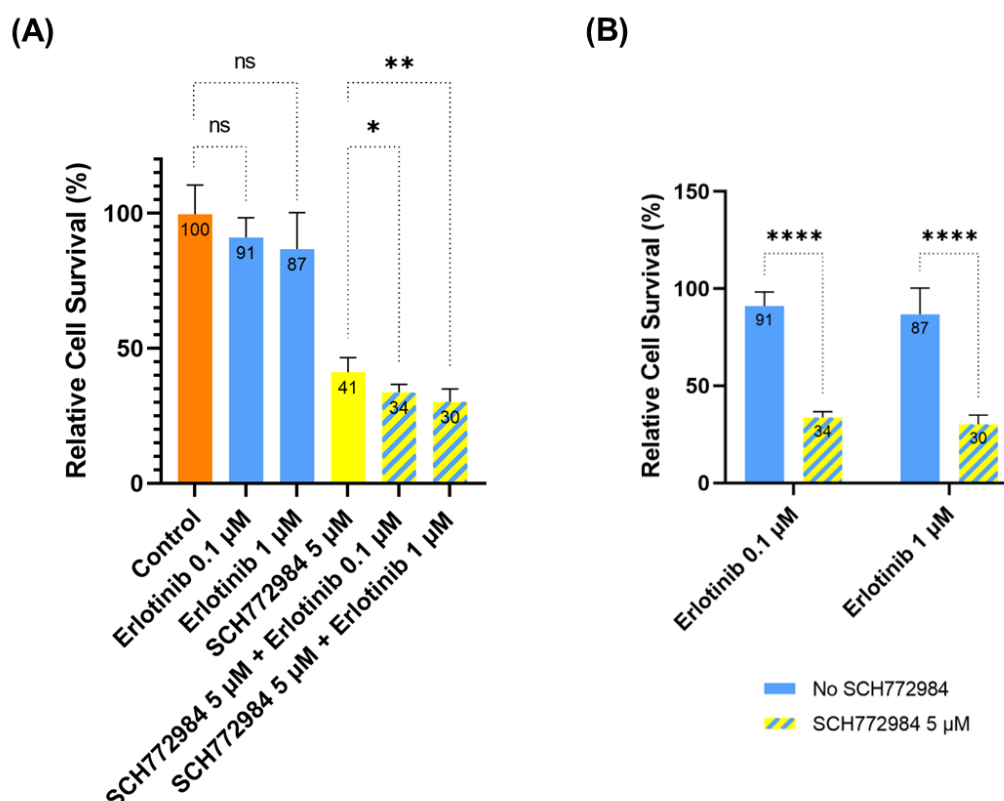


Figure 17 – Erlotinib and SCH772984 combined treatment can resensitize HCC827 cells presenting the resistance phenotype to erlotinib. (A) HCC827 cells that display the resistance phenotype do not show statistically significant differences in survival when treated with 0.1 µM or 1 µM erlotinib in relation to the control condition. Moreover, the combination of erlotinib and 5 µM of SCH772984 causes significant cell death in HCC827 cells exhibiting the resistance phenotype in relation to the SCH772984 condition. (B) Relative cell viability is significantly lower when HCC827 cells presenting the resistance phenotype are treated with the combination of 5 µM SCH772984 and erlotinib compared to erlotinib in monotherapy. Each value is presented as the mean $\pm$ SD (n = 6, six replicates of each condition were used for all assays). Statistical significance was tested using one-way and two-way ANOVA: ns = non-significant (p-value is equal to or greater than 0.05); * = p-value is less than 0.05 and greater than 0.03; ** = p-value is equal to or less than 0.03 and equal to or greater than 0.001; **** = p-value is less than 0.0001.

2. DNA Methylation Analysis

As was previously indicated, the resistance phenotype observed both *in vitro* and *in vivo* is a stable characteristic that is not attributable to common genetic alterations. So, to test the hypothesis that there are molecules secreted from erlotinib-resistant cells that may promote the acquisition of epigenetic modifications in erlotinib-sensitive cells, leading to the resistance phenotype exhibited, a DNA methylation array was performed. To compare the role of DNA methylation in HCC827 cells before and after the acquisition of the resistance phenotype to erlotinib treatment, five DNA samples of HCC827 cells (control) and five DNA

samples of HCC827 cells that display the resistance phenotype were selected for analysis. Additionally, to assess the maintenance of these epigenetic modifications in an *in vivo* setting, three DNA samples harvested from original ERS mice tumors and three DNA samples harvested from retransplanted ERS tumors were analyzed. It was not possible to analyze DNA from ESS tumors due to the lack of DNA harvested from mice' ESS tumors. Consequently, we had to inoculate new mice with HCC827 cells in order to obtain DNA from these tumors for further analysis.

In order to submit the samples for analysis, DNA extraction was performed for *in vitro* samples and DNA quantification was carried out using the Qubit fluorometer for both *in vitro* and *in vivo* samples. The results obtained are illustrated in **Table 2**.

Table 2 – DNA methylation array samples. The concentration, quantity and volume for each DNA sample submitted is listed below. The original ERS tumor and corresponding transplant are displayed using the same color.

		Sample Number	Concentration of dsDNA (ng/ μ L)	Quantity of dsDNA (ng)*	Sample Volume (μ L)**
Mice	Original ERS Tumor	#830	110,0	400	3,64
		#834	102,0	400	3,92
		#837	33,8	400	11,83
	ERS Tumor Transplant	219TE	286,0	400	22,00
		#808	120,0	400	3,33
		#806	15,3	400	26,14
Cell Lines	HCC827 Cell Line	T75 Flask #1	153,0	950	6,21
		T75 Flask #2	48,8	950	19,47
		T75 Flask #3	25,8	950	36,82
		T75 Flask #4	44,2	950	21,49
		T75 Flask #5	39,8	950	23,87
	HCC827 + Conditioned Media + Erlotinib Cell Line	T75 Flask #1	135,0	950	7,04
		T75 Flask #2	144,0	950	6,60
		T75 Flask #3	79,2	950	11,99
		T75 Flask #4	72,2	950	13,16
		T75 Flask #5	93,4	950	10,17

* The quantity of DNA must be between 250 and 500 ng for mice tumors and between 500 and 1000 ng for cell lines.

** Total sample volume must be $\leq 45 \mu$ L.

2.1. There are distinct DNA methylation patterns between HCC827 cells, erlotinib-resistant HCC827 cells and ERS tumor samples

The results of the DNA methylation array, conducted at Hospital Clínico Universitario de Santiago in Spain, were provided to us already preprocessed using the RStudio platform. As such, the methylation status of each CpG analyzed was expressed as β -values, ranging from 0 (indicating no methylation) to 1 (indicating complete methylation).

To allow an overview of the CpG methylation patterns in HCC827 cells, in HCC827 cells that display the resistance phenotype and in original and transplanted mice ERS tumors, a principal component analysis (PCA) plot was generated. Considering that samples that cluster together exhibit high correlation, which indicates similar CpG

methylation patterns, three distinct clusters can be observed in the PCA plot depicted in **Figure 18**: one comprised of the five DNA samples of HCC827 cells, represented as green dots; one consisting in the five DNA samples of HCC827 cells presenting the resistance phenotype, represented as red dots; and the third cluster, although more scattered (with the 219TE transplant sample the furthest away), composed by the original and transplanted ERS tumor samples, represented by orange and yellow dots, respectively.

When comparing the two cell lines (HCC827 cells *versus* HCC827 cells presenting the resistance phenotype), we can see that they cluster far apart, indicating different DNA methylation patterns. However, the second principal component (PC2) axis represents the second largest variation between samples, while the first principal component (PC1) axis represents the greatest variation between samples. This suggests that the difference in DNA methylation between each cell line cluster and the tumors' cluster is greater than the difference in DNA methylation between HCC827 cells and HCC827 cells displaying the resistance phenotype.

Furthermore, the PCA plot showed that PC1 and PC2 account for 32.2% and 14.9%, respectively, of the total variance. This means that the first two principal components together explain 47.1% of the total variation in DNA methylation observed across the three clusters of samples.

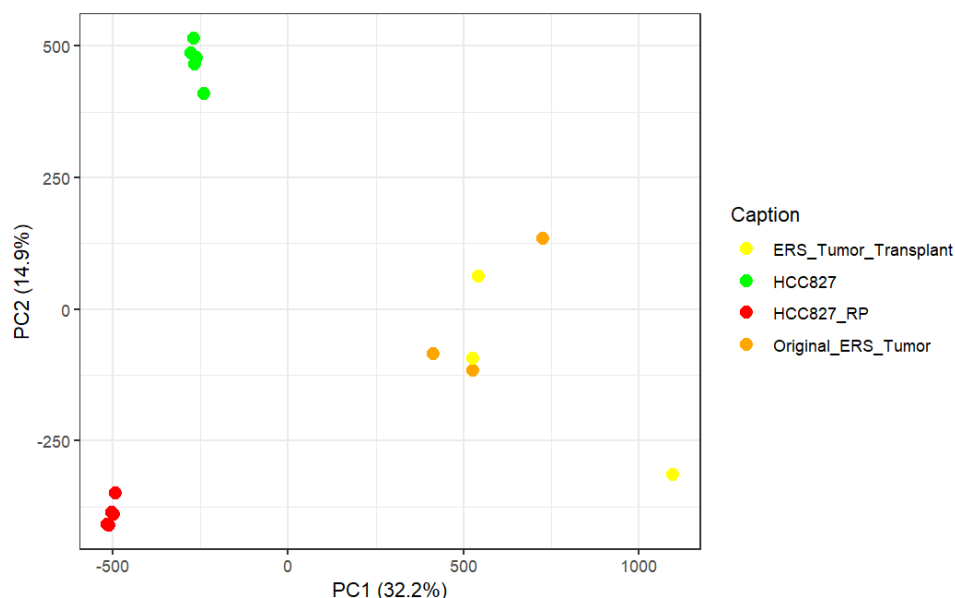


Figure 18 – Principal component analysis (PCA) results. The PC1 (x axis) accounts for 32.2% of the variance in DNA methylation, while the PC2 (y axis) explains 14.9% of this variance. As explained in the caption, each color represents the different *in vitro* and *in vivo* DNA samples analyzed by a DNA methylation array. HCC827_RP = HCC827 displaying the Resistance Phenotype.

2.2. HCC827 cells exhibiting the resistance phenotype are hypomethylated compared to HCC827 cells

The volcano plot in **Figure 19** illustrates the differentially methylated positions (DMPs) between HCC827 cells and HCC827 cells presenting the resistance phenotype. A positive $\log_2$ fold change (FC) indicates that those CpGs (each dot represents a CpG site) are more methylated in HCC827 cells displaying the resistance phenotype compared to HCC827 cells (i.e., they are hypermethylated in HCC827 cells displaying the resistance phenotype). Conversely, a negative $\log_2$ FC indicates that those CpGs are more methylated in HCC827 cells than in HCC827 cells displaying the resistance phenotype (i.e., they are hypomethylated in HCC827 cells displaying the resistance phenotype). Considering this, we can see in **Figure 19** that, overall, HCC827 cells exhibit more methylation in their CpGs than HCC827 cells displaying the resistance phenotype, since there is a higher number of red dots in the left side of the volcano plot than in its right side. Specifically, the CpG with the highest statistically significant fold change between the two cell lines is cg25873934_BC11, with a $\log_2$ FC of ≈ -5.64 . This means that this CpG in HCC827 cells displaying the resistance phenotype is only $2^{-5.64} \approx 0.02 \approx 2\%$ methylated when compared to HCC827 cells (98% of its methylation disappeared). The top 10 CpGs with the highest statistically significant fold change between HCC827 cells and HCC827 cells with the resistance phenotype, and corresponding genes, are represented in **Table 3**.

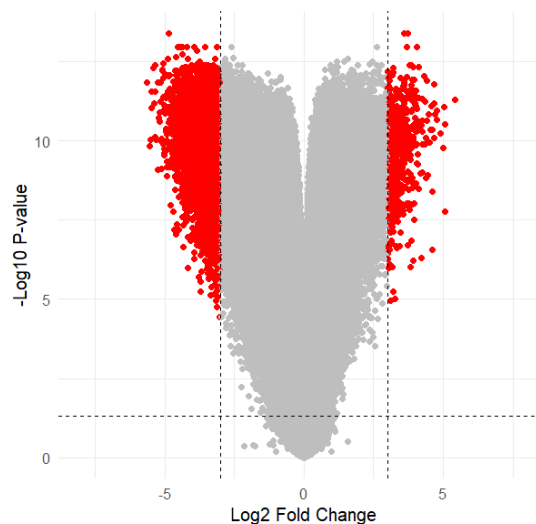


Figure 19 – Volcano plot showing the DMPs between HCC827 cells and HCC827 cells displaying the resistance phenotype. The CpGs highlighted in red are the ones that exhibit the greatest statistically significant differences in DNA methylation between the two cell lines. The horizontal dashed line, representing the $-\log_{10}(\text{FDR adjusted p-value}) = -\log_{10}(0.05)$, allows the identification and exclusion of DMPs that are not statistically significant, shown as gray dots below this line. The two vertical dashed lines, on the other hand, illustrate a $\log_2$ FC greater than 3 and lower than -3. The CpGs that fulfill these two conditions ($3 < \log_2\text{FC} < -3$ and $\text{FDR adjusted p-value} < 0.05$) are highlighted in red.

Table 3 – Top 10 CpGs with the highest statistically significant fold changes in DNA methylation between HCC827 cells and HCC827 cells presenting the resistance phenotype.

CpG Site	FDR Adjusted P-Value	Log ₂ FC	Mean β Values HCC827	Mean β Values HCC827_RP	Chromosome	Position	Relation to Island	Region	Gene Name
cg25873934_BC11	1.43127E-12	-5.637791625	0.503926378	0.010120983	X	11427975	Island	TSS200; TSS1500	ARHGAP6
cg07446674_TC11	1.42958E-10	-5.530362254	0.393313325	0.008510082	X	136251402	Island	TSS200	MAP7D3
cg18221580_TC11	8.87505E-11	-5.507718548	0.568766465	0.012501021	13	48001761	Shore	TSS1500; Exon 1	SUCLA2
cg07419801_BC11	2.70145E-12	-5.488504893	0.430778571	0.0095951	X	132023594	Island	5'UTR	STK26
cg14105985_BC11	9.10983E-12	-5.46095326	0.407326403	0.009247659	9	111036656	Shore	5'UTR	LPAR1
cg00933924_TC11	4.88992E-12	5.438784068	0.011922339	0.517128681	22	36507004	Island	5'UTR	FOXRED2
cg03890843_BC11	4.87023E-13	-5.412418462	0.377166693	0.008855907	X	135973753	Island	Exon 2	MMGT1
cg25870731_TC11	6.49954E-12	-5.365708575	0.349614179	0.008479101	X	141176844	Island	Exon 1	LDOC1
cg10832093_BC11	4.18277E-13	-5.353429399	0.700789568	0.017141341	12	130818649	Island		STX2
cg27069132_TC11	7.3712E-11	-5.343164796	0.398823776	0.009824901	X	136909384	Island	Exon 1	

2.3. The differentially methylated positions (DMPs) of original and transplanted tumors cluster together

The volcano plot in **Figure 20** illustrates the DMPs between the original and transplanted mice ERS tumors. As before, a positive log₂FC indicates that those CpGs (each dot represents a CpG site) are more methylated in the transplanted tumors compared to the original tumors (i.e., they are hypermethylated in the transplanted tumors). Conversely, a negative log₂FC indicates that those CpGs are more methylated in original tumors than in transplanted tumors (i.e., they are hypomethylated in transplanted tumors). Considering this, we can see in **Figure 20** that, overall, the transplanted ERS tumors exhibit slightly less methylation in their CpGs than original ERS tumors, since there is a higher number of red dots in the left side of the volcano plot than in its right side. More importantly, differentially methylated CpGs tend to aggregate around a log₂FC of zero. This indicates that the DNA methylation patterns between the original and transplanted tumors are not highly different, as expected. Specifically, the CpG with the highest statistically significant fold change between the original and transplanted tumors is cg08361262_BC11, with a log₂FC of ≈ 3.88 . This means that this CpG in transplanted ERS tumors is $2^{3.88} \approx 14.7$ times more methylated than in original ERS tumors. The top 10 CpGs with the highest statistically significant fold change between original and transplanted mice ERS tumors, and corresponding genes, are represented in **Table 4**.

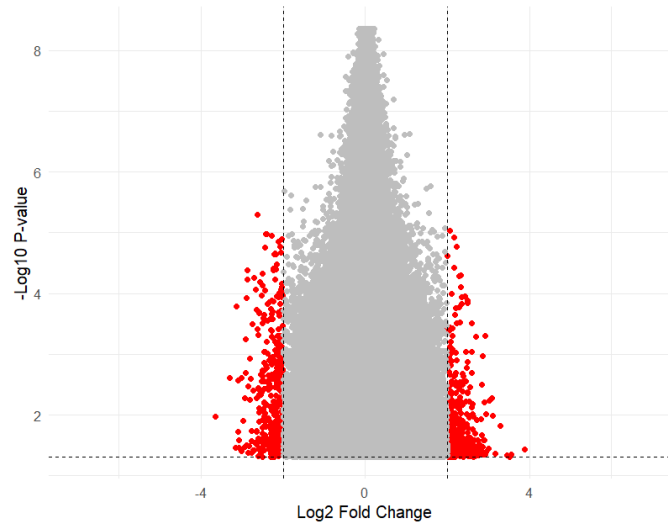


Figure 20 – Volcano plot showing the DMPs between original and transplanted mice ERS tumors. The CpGs highlighted in red are the ones that exhibit the greatest statistically significant differences in DNA methylation between original and transplanted tumors. The horizontal dashed line, representing the $-\log_{10}(\text{FDR adjusted p-value}) = -\log_{10}(0.05)$, allows the identification and exclusion of DMPs that are not statistically significant, shown as gray dots below this line. The two vertical dashed lines, on the other hand, illustrate a $\log_2\text{FC}$ greater than 2 and lower than -2. The CpGs that fulfill these two conditions ($2 < \log_2\text{FC} < -2$ and $\text{FDR adjusted p-value} < 0.05$) are highlighted in red.

Table 4 – Top 10 CpGs with the highest statistically significant fold changes in DNA methylation between original and transplanted mice ERS tumors.

CpG Site	FDR Adjusted P-Value	Log ₂ FC	Mean β Values Original ERS Tumor	Mean β Values Transplanted ERS Tumor	Chromosome	Position	Relation to Island	Region	Gene Name
cg08361262_BC11	0.036548205	3.878138996	0.013781336	0.202641078	5	108381523	Island	Exon 1	FBXL17
cg20219172_TC11	0.010778448	-3.655242808	0.34188054	0.027135427	17	81734448	OpenSea		
cg17815538_BC11	0.044555531	3.561763799	0.016027263	0.189259242	2	237627672	Island	Exon 1	LRRFIP1
cg03143742_TC11	0.0490411	3.51495186	0.019558842	0.223588304	22	20437856	Island	TSS200	SCARF2
cg02957057_TC11	0.046159447	3.44992716	0.019593696	0.214115413	1	236065298	Island	TSS1500	NID1
cg14587604_TC11	0.002435879	-3.309334263	0.288588418	0.029111864	5	159331748	Island	Exon 1	LOC285626
cg14135860_BC11	0.015253688	3.283328238	0.028729078	0.279706123	9	113875776	Shore	TSS1500	ZNF618
cg04696274_BC11	0.034820389	-3.164468735	0.388828047	0.04336682	20	45348891	Shore	TSS1500	SDC4
cg09390080_BC21	0.044069416	3.163918888	0.030506986	0.273422209	1	7479224	OpenSea		
cg10197332_BC21	0.000163627	-3.143138148	0.307738473	0.034833967	6	17268398	OpenSea		

3. CAV1 Knockdown

The expression of the *CAV1* gene was 7.7-fold higher in ERS tumors when compared to ESS tumors in previous RNA-seq results analyzed using the “KEGG pathways” platform (Junqueira-Neto et al., 2022). Therefore, in order to validate the role of *CAV1* and caveolae-dependent endocytosis in the acquisition of the resistance phenotype to erlotinib treatment, we attempted to establish a *CAV1* knockdown cellular model. For this, HCC827 cells were transduced using a lentiviral vector which included a shRNA to reduce *CAV1* gene expression or a scrambled sequence, a puromycin resistance marker to positively select antibiotic-resistant cells and a tGFP reporter gene to assess successful transduction. Upon the formation and growth of puromycin resistant colonies, the transduced cells were collected and stained with an anti-CAV1 antibody conjugated with the

PE fluorophore (red fluorescence) which was detected by flow cytometry to confirm whether the *CAV1* knockdown was successful. Non-transduced *CAV1*-unstained HCC827 cells and non-transduced *CAV1*-stained HCC827 cells were used as controls.

The results obtained are displayed in **Figure 21**, where the x axis represents the green fluorescence from tGFP expression by successfully transduced cells detected by the fluorescein isothiocyanate (FITC) channel, and the y axis represents the red fluorescence from the anti-*CAV1* antibody staining detected by the PE channel.

As expected, the FITC and PE channels detected no fluorescence produced by non-transduced *CAV1*-unstained HCC827 cells (first panel). Non-transduced HCC827 parental cells (second panel), on the other hand, do not express tGFP (since they were not transduced) and so there is no fluorescence detected by the FITC channel. However, the PE channel detects red fluorescence in these cells due to the staining with the anti-*CAV1* antibody. This provides the basal *CAV1* levels of these cells, showing that 99.4% of all non-transduced HCC827 parental cells express *CAV1*.

The results obtained with the HCC827 cells transduced with the scramble (third panel) and with construct #1 (fourth panel) allowed the identification of three populations of transduced cells: cells that do not express tGFP and so do not present green fluorescence (tGFP⁻ cells); cells with a dim tGFP expression detected by the FITC channel (tGFP^{dim} cells); and cells that highly express tGFP (tGFP^{high} cells). Regarding HCC827 cells transduced with the scramble (third panel), we can see that the three populations of cells – 8.97% of tGFP⁻ cells, 80.4% of tGFP^{dim} cells and 10.3% of tGFP^{high} cells – consistently express *CAV1* as the tGFP fluorescence increases. This is expected since the scramble sequence does not reduce *CAV1* gene expression. Moreover, when looking at the HCC827 cells transduced with construct #1, the three populations of cells – 19.6% of tGFP⁻ cells, 77.3% of tGFP^{dim} cells and 3.55% of tGFP^{high} cells – exhibit a *CAV1* expression that tends to decrease as the tGFP fluorescence increases.

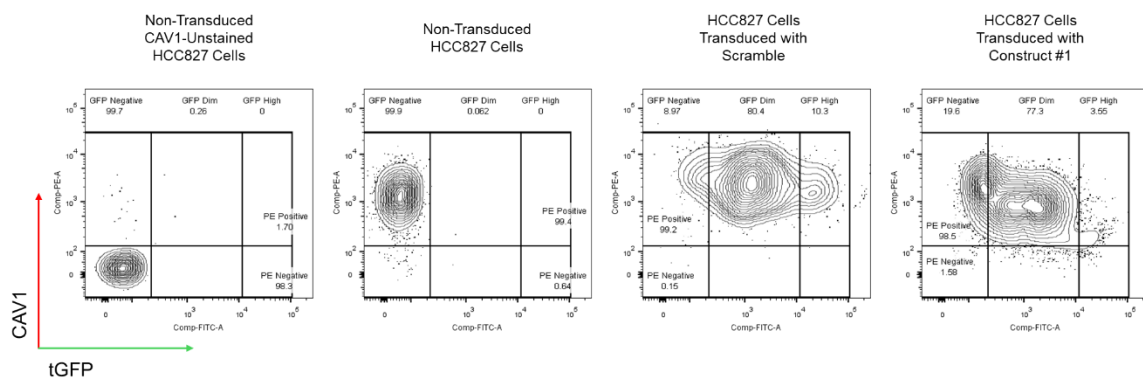


Figure 21 – Flow cytometry analysis of tGFP and *CAV1* expression in transduced HCC827 cells. The FITC channel, represented on the x axis, detects tGFP expression, while the PE channel, represented on the y axis, detects the anti-*CAV1* antibody fluorescence. From left to right, the panels display the flow cytometry results as contour plots for non-transduced *CAV1*-unstained HCC827 cells, non-transduced *CAV1*-stained HCC827 cells, HCC827 cells transduced with the scramble and HCC827 cells transduced with construct #1.

The bar graph in **Figure 22A** represents the mean fluorescence intensity (MFI) of the PE fluorophore (conjugated with the anti-CAV1 antibody), providing an overview of the *CAV1* expression across the four cellular conditions described above. Again, non-transduced CAV1-unstained HCC827 cells virtually produce no red fluorescence detected by the PE channel. Non-transduced HCC827 parental cells and HCC827 cells transduced with the scramble exhibit basal *CAV1* levels, with a higher *CAV1* expression in HCC827 cells transduced with the scramble compared to the non-transduced cells. Moreover, it is evident that there is not a substantial difference in the PE fluorescence, and therefore in *CAV1* expression, between non-transduced HCC827 cells and those transduced with construct #1.

A closer look at HCC827 cells transduced with construct #1, depicted in **Figure 22B**, shows that the tGFP⁻ cells, which did not incorporate the lentiviral vector, show a higher expression of *CAV1* than both tGFP^{dim} and tGFP^{high} cells, which successfully incorporated the vector. Additionally, tGFP^{dim} cells exhibit a higher expression of *CAV1* compared to tGFP^{high} cells.

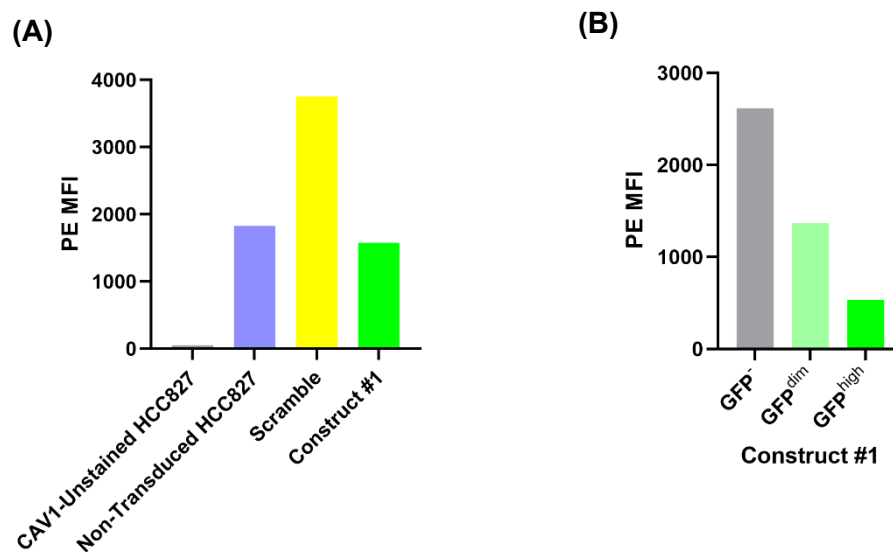


Figure 22 – Bar graphs illustrating the mean fluorescence intensity (MFI) of the PE fluorophore across all cellular conditions (A) and in a detailed view of construct #1 (B). The PE fluorophore is conjugated with the anti-CAV1 antibody and, therefore, indicates the *CAV1* gene expression in each cellular condition.

Discussion

In this study, we aimed to explore the underlying mechanisms of erlotinib resistance in LADC cells and investigate potential strategies to overcome this resistance. The development of resistance to EGFR-TKI therapies, such as erlotinib, remains a significant challenge in the treatment of lung cancer. Our findings highlight the critical role of CAV1-mediated signaling and the dysregulation of key pathways involving PI3K, AKT, and ERK in promoting resistance phenotypes. Additionally, our DNA methylation analysis revealed distinct epigenetic alterations between erlotinib-sensitive and resistant cells, suggesting that these modifications may further contribute to the stability and persistence of the resistance phenotype. This discussion will focus on the implications of these findings, particularly the potential of targeting these pathways and epigenetic changes to resensitize resistant cells to the EGFR-TKI therapy.

Previous results from our group revealed an upregulation of PI3K and an increased activation of AKT1 and ERK1/2 in ERS tumors from mice, compared to ESS tumors, likely due to the upregulation of *CAV1*, resulting in the goal described in objective 1. So, HCC827 cells supplemented with CM from H1975 cells and treated with erlotinib, which display the erlotinib-resistant phenotype mentioned before, were treated with the PI3K inhibitor wortmannin, with the AKT inhibitor miransertib and with the ERK inhibitor SCH772984 in order to analyze if the combination treatment of erlotinib and each of these drugs resensitizes these cells to the EGFR-TKI therapy. For this analysis, MTT assays were performed using erlotinib-sensitive HCC827 cells and HCC827 cells that exhibit the resistance phenotype.

Regarding the PI3K inhibitor wortmannin results, we can see that there is a decrease in HCC827 cells' viability that reaches around 65% with a concentration of 5 μM of wortmannin in the IC₅₀ assay. One explanation for this is that PI3K is activated downstream of mutant *EGFR* and not only as a resistance mechanism to erlotinib. At a certain concentration, cell death can be explained by drug cytotoxicity. Furthermore, when HCC827 cells are treated with 0.1 μM or 1 μM of erlotinib, cell survival falls from 92% to around 42%, which is expected since these cells are sensitive to erlotinib therapy. Moreover, with the dual inhibition of EGFR and PI3K, through the employment of erlotinib and wortmannin respectively, there is not a statistically significant difference in cell viability when compared to solely EGFR inhibition through the employment of erlotinib. This somehow suggests that HCC827 cells are more sensitive to erlotinib therapy than to the combination with wortmannin. Even though this drug affects cell viability, as seen in the IC₅₀ assay, it does not appear, in this case, to be leading to increased cell death through alternative mechanisms downstream of EGFR activation.

HCC827 cells that present the resistance phenotype maintain a cell viability close to 100% when treated with erlotinib, highlighting their resistance to this EGFR-TKI. The dual inhibition of EGFR and PI3K makes cell viability fall to approximately 33%. This indicates that the combination treatment of erlotinib and wortmannin can resensitize HCC827 cells presenting the resistance phenotype to erlotinib therapy. It also confirms that the PI3K signaling pathway is activated in LADC cells that acquire the resistance phenotype. This was also verified in a study by (Zhou et al., 2021) which showed that the dual inhibition of EGFR (with the EGFR-TKI erlotinib) and PI3K (with LY294002) also significantly decreased cell viability in NSCLC cell lines harboring wild-type *PIK3CA* (our previous results showed no mutations in *PIK3CA* in ERS tumors), when compared to erlotinib or LY294002 monotherapy.

Now focusing on the AKT inhibitor miransertib, when we look at the MTT assay results for HCC827 cells using 0.1 μM and 1 μM of erlotinib, there is a statistically significant decrease in cell viability, as expected due to the cells' sensitivity to erlotinib therapy, with the cell viability dropping to around 42% with erlotinib treatment. Moreover, when HCC827 cells are treated with both erlotinib and miransertib there is also a significant decrease in relative cell survival in comparison to miransertib alone. When comparing both conditions (erlotinib *versus* erlotinib plus miransertib), there is a statistically significant difference with the HCC827 cells treated with erlotinib in monotherapy showing less survival. This suggests that adding miransertib to the erlotinib treatment in erlotinib-sensitive cells does not significantly change relative survival, which means that the death of these cells is mainly due to the EGFR-TKI. Supporting this is that, in previous IC50 assays, HCC827 cells maintain their viability with increasing concentrations of miransertib, with their relative survival surrounding 100%. Nonetheless, this could be due to the fact that cell lines carrying *PIK3CA* or *PIK3R1* mutations are more sensitive to miransertib than the ones that are wild-type for these genes, which is the case of HCC827 cells (Yu et al., 2015).

Furthermore, in the MTT assay results, HCC827 cells presenting the resistance phenotype do not show a statistically significant difference in cell viability when treated with erlotinib, which is in accordance with their resistance to this EGFR-TKI therapy. Nevertheless, there is not a significant change in relative cell survival when these cells are treated with both erlotinib and miransertib, demonstrating no differences in cell viability with the addition of the AKT inhibitor. This is not consistent with what was seen in previous IC50 assays, where 13 μM of miransertib corresponded to approximately 85% of HCC827 cells displaying the resistance phenotype alive. This lack of efficacy was also evidenced with 9 μM and 20 μM of miransertib in additional MTT assays. Moreover, a study by (Lefebvre et al., 2023) demonstrated that the dual inhibition of EGFR, with erlotinib, and AKT, with the allosteric pan-AKT inhibitor VIII, inhibits the proliferation of triple-negative breast cancer

cells in more than 50% and more than 70% when using 1 μ M of AKT inhibitor VIII and either 0.1 μ M or 1 μ M of erlotinib, respectively. All of this suggests that either miransertib is not an adequate AKT inhibitor for this cell line, as it fails to demonstrate a consistent and coherent activity, or that effectors downstream of AKT are being activated by other kinases and promoting cell survival either way. An example is GSK-3, which can be phosphorylated and consequently inactivated not only by AKT, but also by RSK (downstream of ERK), resulting in cell survival and proliferation (Manning & Toker, 2017). Another explanation to the lack of efficacy of miransertib could be the presence of mutations in *AKT1* (e.g. E17K) in this cell line, making these cells resistant to the drug's effect. However, according to (Yu et al., 2015), miransertib works irrespective of mutations in *AKT*.

Nevertheless, this observation raises the question of why the PI3K inhibitor significantly reduced the survival of LADC cells exhibiting the resistance phenotype, while the AKT inhibitor did not alter the relative survival of the same HCC827 cells with the resistance phenotype, if the underlying cause is the activation of downstream compensatory mechanisms. One reason for this is that wortmannin is a non-specific PI3K inhibitor and so, besides inhibiting PI3K, it also targets structurally similar molecules, such as the mammalian target of rapamycin (mTOR). Moreover, the inhibition of PI3K prevents PDK1 activity, which is responsible for phosphorylating and activating several kinases, namely PKC, S6K, SGK, and RSK. These kinases can then phosphorylate multiple targets downstream of AKT, which are also phosphorylated by AKT (e.g., GSK-3 can be phosphorylated by PKC and S6K, FOXO by SGK) (Manning & Toker, 2017). This could explain why the PI3K inhibitor (wortmannin) was effective but the AKT inhibitor (miransertib) was not.

Lastly, regarding the ERK inhibitor SCH772984 results, when HCC827 cells are treated with erlotinib their viability significantly drops from 106% to around 46%, reinforcing the sensitivity of these cells to the EGFR-TKI. Moreover, when these cells are treated with erlotinib and SCH772984, there is a statistically significant decrease in cell viability from 55% to around 33%. When comparing the treatment of erlotinib *versus* erlotinib plus SCH772984, we can see that the differences in relative cell survival caused by these treatments are significantly different, with cell death being higher with the administration of erlotinib and SCH772984. This means that, in HCC827 cells, the addition of the ERK inhibitor SCH772984 to the erlotinib treatment causes even more cell death, probably because the Raf-MEK-ERK signaling pathway is more activated in these erlotinib-sensitive cells.

Furthermore, the treatment of HCC827 cells presenting the resistance phenotype with erlotinib causes no significant changes in relative cell survival due to the already mentioned therapy resistance. However, when the ERK inhibitor SCH772984 is added to

the erlotinib treatment, cell viability significantly drops from 41% to around 32%. Therefore, when comparing the treatments erlotinib *versus* erlotinib plus SCH772984, we can see that there is a statistically significant reduction in the relative survival of HCC827 cells presenting the resistance phenotype. This suggests that adding this ERK inhibitor to the erlotinib treatment in cells resistant to erlotinib could potentially restore their sensitivity to the EGFR-TKI and further confirms the activation of this kinase in HCC827 cells exhibiting the resistance phenotype. This was consistent with the results of a study conducted by (Ku et al., 2022) in which PC9/ER erlotinib-resistant cells, also lacking the T790M mutation but presenting a missense mutation in *NRAS* that led to ERK activation (no point mutations in *KRAS*, *NRAS* and *BRAF*, as well as CNVs in *BRAF*, were detected in our model), treated with both erlotinib and the selective ERK1/2 inhibitor ASN007 showed less cell viability than each drug in monotherapy.

Finally, to validate our findings regarding the inhibition of PI3K, AKT and ERK, it is essential to perform further experiments. Western blots should be used to confirm that the observed decrease in cell viability is indeed due to the inhibition of the pathways involving these targets and not cytotoxicity of the drugs. This technique can also be used to analyze if there is a reduction in the phosphorylation of molecules downstream of these targets. Moreover, MTT assays measure cell viability at a single time point so, to overcome this, colony-forming assays can be performed to assess if the inhibitory effects of erlotinib combined with these drugs are sustained over time.

Ultimately, our findings are promising because, even though they are in an early stage, they can be the future of targeted therapies in lung cancer. They can potentially delay the onset of drug resistance or even prevent its occurrence, meaning that it can improve the efficacy of lung cancer treatments.

Regarding objective 2, previous *in vitro* and *in vivo* experiments performed by our group proved that the erlotinib resistance developed is a stable phenotype that is not dependent on the acquisition of commonly observed resistance mutations in LADC. Hence, we hypothesized that molecules transferred between erlotinib-resistant cells and erlotinib-sensitive cells promote the acquisition of epigenetic modifications in the latter, resulting in the resistance phenotype observed. As a first approach to this research hypothesis, we decided to analyze the DNA methylation patterns between HCC827 cells before and after the acquisition of resistance to erlotinib treatment and between original and transplanted ERS tumors from mice, using a DNA methylation array.

Overall, the results of this DNA methylation array allowed the observation that the DNA methylation patterns between LADC cells sensitive to erlotinib (HCC827 cells) and LADC cells that develop the resistance phenotype (HCC827 cells that exhibit the resistance

phenotype, as well as original and transplanted ERS tumors from mice) are different, showing a decrease in DNA methylation with the progression of the cancer.

Regarding the PCA results, it is evident that the five DNA samples from HCC827 cells cluster together, as do the five DNA samples from HCC827 cells displaying the resistance phenotype. This is in accordance with the fact that these DNA samples, which were submitted to the DNA methylation array, are technical replicates and, therefore, the DNA methylation patterns between them should not vary extensively. Moreover, while it was possible to identify a cluster comprising both the original and transplanted ERS tumors, their DNA methylation patterns were not as closely related to each other as those observed within each of the individual cell line clusters. This is also supported by the volcano plot of DMPs between the original and transplanted ERS tumors. Additionally, the large dispersion, and thus, high variation among these tumor samples leads to less variance explained (Gonzalez et al., 2023), which justifies why the first two principal components (PC1 and PC2) only explain 47.1% of the total variance in DNA methylation among the three clusters of samples.

Now focusing on the volcano plot illustrating the statistically significant fold changes in DNA methylation between HCC827 cells and HCC827 cells presenting the resistance phenotype. It is possible to see that HCC827 cells exhibit more methylation in their CpGs than HCC827 cells displaying the resistance phenotype. This suggests that, with the acquisition of the resistance phenotype, the DNA from LADC cells becomes more hypomethylated. Similar results have already been described by (Guo et al., 2013) when comparing the methylation status of gene promoters between A549 LADC parental cells and A549 cells resistant to cisplatin (A549/DDP) using methylated-DNA immunoprecipitation-chromatin immunoprecipitation (MeDIP-ChIP). Within the differentially methylated promoters identified, around 56.3% were found hypomethylated in A549/DDP cells while the remaining 43.7% were hypermethylated. Furthermore, when the hypomethylation occurs in CpG islands, CpG islands shores or in the first exon, the associated gene (usually an oncogene), which was previously silenced by the epigenetic modification, is now reactivated (Moore et al., 2013).

When examining the table of the top 10 CpGs with the highest statistically significant fold changes in DNA methylation between HCC827 cells and HCC827 cells presenting the resistance phenotype, while some genes, such as *SUCLA2*, *FOXRED2*, *MMGT1* and *STX2*, have not yet been associated with DNA methylation and therapy resistance in cancer, others have been related to therapy resistance in several cancer types, like *MAP7D3* (chemoresistance in breast cancer (Kuo et al., 2023)), *LPAR1* (targeted therapy resistance in melanoma (Liu et al., 2021) and chemoresistance in breast cancer (Chou et al., 2021)), *STK26* (radioresistance in glioblastoma (Huang et al., 2017)) and *ARHGAP6* (bevacizumab resistance in endometrial cancer (Davies et al., 2011)). Only *STK26* was associated with

DNA methylation: irradiation reduces the methylation of the *STK26* promoter, allowing the phosphorylation and activation of the autophagy related 4B cysteine peptidase (ATG4B) and consequent radioresistance in glioblastoma (Huang et al., 2017).

Additionally, STK26 can activate the ERK and AKT signalling pathways and mediate the epithelial-mesenchymal transition (EMT), which has already been associated with therapy resistance (Getu et al., 2023; Li et al., 2019). Although it is known that STK26 can activate the ERK and AKT signalling pathways, the intermediate molecules involved in these processes are not known (Thompson & Sahai, 2015). What is known is that ERK activation is independent of Ras-Raf activation (Lin et al., 2001). In fact, STK26 can directly phosphorylate and activate ERK or it can interact with programmed cell death 10 (PDCD10) to phosphorylate and activate ERK (Zhou et al., 2024). ERK then leads to the transcription and upregulation of mesenchymal markers, such as N-cadherin and vimentin, and downregulation of epithelial markers, such as E-cadherin (Li et al., 2019; Lin et al., 2014). Moreover, STK26 interacts and phosphorylates ezrin on threonine 567, leading to the activation of AKT. This facilitates EMT also due to the upregulation of mesenchymal markers and downregulation of epithelial markers (Arora et al., 2022; Yu et al., 2017). These two signalling pathways trigger EMT and conduct therapy resistance. Although the mechanisms behind this remain unclear, it is believed that they are associated with the expression of antiapoptotic genes, such as those activated downstream of the PI3K-AKT and Raf-MEK-ERK pathways (Song & Faber, 2019; Yu et al., 2017).

In addition, when looking at the volcano plot illustrating the statistically significant fold changes in DNA methylation between original and transplanted ERS tumors, we can see that, even though there are significant differences in DNA methylation between the original and transplanted tumors, the majority of the differentially methylated CpGs tend to aggregate around a $\log_2FC$ of zero. As was previously said, this indicates that the DNA methylation patterns between the original and transplanted tumors are not highly different, which is in accordance with our observation that the changes undergone by the original tumor are stable, resulting in a tumor with similar characteristics upon being transplanted onto new mice. Furthermore, when examining the table of the top 10 CpGs with the highest statistically significant fold changes in DNA methylation between original and transplanted ERS tumors from mice, none of the associated genes were linked to the development of targeted therapy resistance via DNA methylation.

Finally, considering both the PCA results and the insights provided by the two volcano plots, it is possible to conclude that indeed there are differences in the DNA methylation patterns of LADC cells sensitive to erlotinib therapy and the ones that develop the resistance phenotype. In fact, this resistance phenotype is characterized by an overall decrease in DNA methylation and subsequent increase in gene expression (when the

methylation occurs in CpG islands, CpG islands shores or first exon). Therefore, molecules secreted from erlotinib-resistant cells indeed affect the DNA methylation status of erlotinib-sensitive cells, contributing to the development of their resistance phenotype.

Ultimately, even though the DNA methylation array provided a thorough overview of the methylation patterns between the conditions studied, it is important to remember that other epigenetic modifications, such as histone post-translational modifications (e.g., acetylation) and non-coding RNAs, can also affect the cells' DNA transcription (Moore et al., 2013) and are therefore worth exploring.

Finally, concerning objective 3, our group previously found that, when comparing ERS and ESS tumors using RNA-seq, the expression of *CAV1* is 7.7-fold higher in ERS tumors than in ESS tumors. This led us to hypothesize that this caveolae-associated molecule was upstream of the activation of signaling pathways that led to the resistance phenotype observed in ERS tumors, namely the PI3K-AKT pathway and the Raf-MEK-ERK pathway. Hence, with the ultimate aim of validating the role of *CAV1* in the acquisition of the resistance phenotype to erlotinib treatment, we attempted to establish a *CAV1* knockdown cellular model using lentiviral vectors containing a shRNA that reduced *CAV1* gene expression.

Many techniques could have been used to achieve *CAV1* gene knockdown besides viral transduction, such as nonviral transfection and clustered regularly interspaced short palindromic repeats (CRISPR) interference (CRISPRi). Among the most commonly used viral vectors for transduction, lentivirus was the appropriate choice considering that adenovirus only cause transient transduction and that adeno-associated virus (AAV) are usually used to transduce slow dividing cells. However, the main disadvantage of using viral vectors to perform gene knockdown is the fact that their random insertion into the host cell genome may cause mutations (Butt et al., 2022). A method that overcomes this problem is CRISPRi. CRISPRi uses deactivated/dead Cas9 (dCas9) fused with a single guide RNA (sgRNA), complementary to regions downstream of the transcription start site (TSS) of the gene of interest, and fused with a transcriptional repressor, such as the Krüppel associated box (KRAB), which promotes gene repression through chromatin condensation, to accomplish gene knockdown (Shakirova et al., 2020). This is a very effective and specific method, but it still has its limitations, such as the possibility that preventing the transcription of a target gene blocks the transcription of downstream genes (polar effect) (Zhang et al., 2021). Finally, although nonviral vectors allow stable gene repression, they are exposed to extracellular and intracellular (e.g., lysosomes) barriers that cause low transfection efficiency (Butt et al., 2022). Considering that none of these methods are excellent, we chose to perform viral transduction.

Regarding the negative controls used for the flow cytometry of transduced HCC827 cells, the HCC827 cells that were neither transduced nor stained with the anti-CAV1 antibody exhibited negative PE (which detects the antibody fluorescence) and FITC (which detects the tGFP expression due to successful transduction) fluorescence; and the HCC827 cells that were not transduced exhibited a negative tGFP expression, as expected, and a positive *CAV1* expression detected in the PE channel and showcasing that 99.4% of HCC827 cells express *CAV1*. In addition to a positive control for PE, a positive control for tGFP should also have been included, such as using HCC827 cells transduced with the scramble not stained with the anti-CAV1 antibody.

The scrambled sequence was successfully incorporated into the genome of $80.4\% + 10.3\% = 90.7\%$ HCC827 cells as confirmed by the dim and high tGFP fluorescence. Regarding the PE fluorescence, which translates into the expression of *CAV1*, these HCC827 cells transduced with the scramble show a constant positive red fluorescence, indicating that these cells are expressing *CAV1*, as expected. In fact, it is possible to compare the anti-CAV1 antibody fluorescence levels of these cells to those of non-transduced HCC827 parental cells, with these cells presenting an even higher *CAV1* expression than the one from non-transduced cells.

Finally, the lentiviral construct #1 was successfully incorporated into the genome of $77.3\% + 3.55\% = 80.85\%$ HCC827 cells as confirmed by the dim and high GFP fluorescence. Additionally, regarding the *CAV1* expression (observed through the PE fluorescence), these cells exhibit lower *CAV1* levels as the tGFP fluorescence increases. This means that tGFP⁻ cells, which did not incorporate the lentiviral vector, show a higher expression of *CAV1* than tGFP^{dim} cells, which incorporated the lentiviral vector and present a reduced *CAV1* gene expression (the *CAV1* knockdown is occurring). In fact, it is possible to compare the *CAV1* fluorescence levels of tGFP⁻ HCC827 cells transduced with construct #1 to those of non-transduced HCC827 cells. Moreover, tGFP^{high} cells successfully incorporated the lentiviral vector and present an even more reduced *CAV1* expression than tGFP^{dim} cells. However, within the successfully transduced cells (tGFP^{dim} + tGFP^{high} cells), a large proportion continue exhibiting high expression of *CAV1* and, as such, do not yet show a successful *CAV1* knockdown. As this is an ongoing work, the next step will be to sort and culture these tGFP^{high} HCC827 cells transduced with construct #1, as an optimization step, and to re-test the other three constructs, which yielded inconclusive results (construct #2) or were unsuccessfully incorporated (constructs #3 and #4).

Although we were unable to validate the role of *CAV1* in the acquisition of the resistance phenotype, since it requires further experiments, we established a *CAV1* knockdown cellular model, which still needs some improvements. This will allow, in the future, for the better comprehension of the signaling pathways downstream of *CAV1* that

are being activated and resulting in the erlotinib's resistance phenotype. Therefore, we intend to supplement the *CAV1* knockdown HCC827 cells with CM from erlotinib-resistant H1975 cells and subsequently treat them with erlotinib in order to verify if the resistance phenotype occurs or takes longer to develop. Furthermore, we also plan to carry out western blots as further validation that the expression of *CAV1* is indeed reduced in the cellular model. Moreover, the phosphorylation status of proteins found to have increased phosphorylation in ERS tumors when compared to ESS tumors, such as AKT1 and ERK1/2, will be analyzed using the *CAV1* knockdown cells in order to provide insights regarding the affected mechanisms downstream of *CAV1* and identify mechanisms mediated by *CAV1* in the acquisition of the resistance phenotype.

Conclusion and Future Perspectives

For this project, we aimed to explore how the overexpression of *CAV1* contributes to the development of resistance to EGFR-TKI therapies in LADC. To achieve this, drugs targeting the kinases PI3K, AKT and ERK – which were found upregulated in mice tumors that exhibit the resistance phenotype – were tested in combination with erlotinib using MTT assays to assess their ability to restore sensitivity to the EGFR-TKI. The EGFR-PI3K and EGFR-ERK dual inhibition rescued sensitivity to erlotinib in LADC cells that exhibit the mentioned resistance phenotype. Hence, considering that both these drugs rescued some sensitivity to erlotinib treatment and that they are intertwined in the regulation of cell survival, it is worth exploring the combination of erlotinib, wortmannin and SCH772984 in HCC827 cells displaying the resistance phenotype, in order to determine if the relative cell survival could be further reduced. Moreover, to validate the results obtained with the multiple MTT assays conducted by us, western blots should be performed. This will allow us to determine whether the mechanism of action of the selected drugs is functioning as expected and therefore reducing the phosphorylation of the target molecules. Furthermore, these combination treatments can be tested in our mouse models to check their efficacy *in vivo* but also to assess potential toxicities they may cause.

Next, for a better understanding of the differences between HCC827 cells before and after acquiring the erlotinib-resistant phenotype, a DNA methylation array was performed. This allowed the discovery of differentially methylated CpG sites when comparing HCC827 cells to HCC827 cells presenting the resistance phenotype, as well as when comparing original ERS tumors to transplanted ERS tumors (although to a lesser extent). Overall, these differences were characterized by a hypomethylation of CpGs as the resistance phenotype developed. Despite the identification of CpGs with the greatest differences in DNA methylation between cell lines and tumors, further investigation is needed to determine the relevance of each gene in the acquisition of this resistance phenotype. Furthermore, complementary experiments, such as chromatin immunoprecipitation sequencing (ChIP-Seq), should be performed to determine how post-translational modifications of histones influence the development of this erlotinib-resistant phenotype.

Moreover, we performed the transduction of HCC827 cells to validate the role of *CAV1* in the acquisition of the resistance phenotype to erlotinib treatment. We established the *CAV1* knockdown cellular model, although it still needs some improvements, so that future experiments can be performed in it, such as supplementing the *CAV1* knockdown cells with CM from H1975 cells and subsequently treat them with erlotinib in order to verify if the resistance phenotype occurs or takes longer to develop. Furthermore, we also plan to

carry out western blots to validate the reduction of *CAV1* expression in this cellular model, as well as to validate the decrease of AKT1 and ERK1/2 phosphorylation, since these kinases presented increased phosphorylation in tumors displaying the resistance phenotype compared to erlotinib-sensitive S (ESS) tumors. Furthermore, we also aim to establish a cellular model for *CAV1* upregulation to further validate the role of this protein in the erlotinib-resistant phenotype.

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